

ANTIMICROBIAL STRATEGIES AGAINST
BURKHOLDERIA CEPACIA

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ABBREVIATIONS

AGE	Aqueous garlic extract
ALP	Antileukoprotease
ASF	Airway surface fluid
ATCC	American type culture collection
ATP	Adenosine 5' triphosphate
BCESM	<i>Burkholderia cepacia</i> epidemic strain marker
bp	Base pair
ceftaz	Ceftazidime
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
cfu	Colony forming units
CGD	Chronic granulomatous disease
chlor	Chloramphenicol
CIA	Cepacia isolation agar
cipro	Ciprofloxacin
colo	Colomycin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphates
DTDP	4,4'-dithiodipyridine
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
EnaC	Epithelial sodium channel
ET12	Electrophoresis type 12
grepa	Grepafloxacin
hBD	Human β -defensin
HD	Human α -defensin
HDL	High density lipoprotein
HNE	Human neutrophil elastase
HNP	Human neutrophil α -defensin
HPLC	High performance liquid chromatography
IL-8	Interleukin 8
ISA	Iso-sensitest agar
ISB	Iso-sensitest broth

kb	Kilobase
KDO	3-deoxy-d-manno-octulosonic acid
LB	Luria broth
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
mBD	Murine β -defensin
mero	Meropenem
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
NADPH	Phosphorylated nicotinamide adenine dinucleotide
NCTC	National collection of type cultures
NO	Nitric oxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PIA	Pseudomonas isolation agar
PLC	Phospholipase C
poly	Polymyxin
psi	Pounds per square inch
RBGE	Royal Botanic Gardens Edinburgh
rifamp	Rifampicin
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RP-HPLC	Reverse phase high performance liquid chromatography
-SH	Sulphydryl
SLPI	Secretory leukoprotease inhibitor
TAP	Tracheal antimicrobial peptide
tobra	Tobramycin
trimeth	Trimethoprim
vanco	Vancomycin

ABSTRACT

Burkholderia cepacia was first identified in the 1950's as a phytopathogen, in particular as the causal agent of soft rot disease in onions. In the 1970's, *B. cepacia* became recognised as a cause of life-threatening pulmonary infection in human hosts, in particular patients in intensive care units and individuals with the inherited disorders chronic granulomatous disease and cystic fibrosis (CF).

Pulmonary colonisation by *B. cepacia* is a particular problem in CF patients since 20% of those colonised succumb to 'cepacia syndrome', a rapidly fatal necrotising pneumonia sometimes accompanied by septicaemia. Evidence from national CF databases also shows that colonisation reduces life expectancy by 50%. Epidemic strains are readily transmitted between patients both nosocomially and through social contact. A further problem is that *B. cepacia* is inherently resistant to most classes of antibiotics and thus infections are difficult to treat. Several mechanisms of resistance have been identified in *B. cepacia* including reduced permeability to most classes of antibiotics and production of highly inducible β -lactamases.

The aim of this project was to investigate novel antimicrobial strategies against *B. cepacia*, based on natural antimicrobial compounds present in plants and human airways as protection against bacterial disease. The project focused on a panel of 20 strains of the *B. cepacia* complex. Isolates included strains representing major epidemic clones of *B. cepacia* and exhibiting a range of susceptibilities to all classes of conventional antibiotics. The project is focused on two major themes. First, a study of antimicrobials from plants and second, examination of the antimicrobial activities of cationic peptides present in human airway secretions.

The susceptibility of the *B. cepacia* strain panel to plant extracts was investigated, in particular the activity of aqueous garlic extract (AGE) and thyme oil. The MIC of thyme oil for all 20 strains was found to be 0.01%. MICs of AGE ranged from 0.25%-3%. Killing curves suggested that AGE produces a slow killing effect over a twenty hour period, whereas thyme oil kills bacterial cells in less than 20 minutes. By electron microscopy, no intact bacteria were observed after three minutes incubation with thyme oil. In contrast, two hours incubation with AGE produced morphological changes in the cellular structure of *B. cepacia* consistent with much slower damage to the bacterial cell membrane. Attempts were then made to purify and identify the chemical nature of the antimicrobial agents using reverse phase HPLC. AGE was shown to contain allicin along with other anti-*cepacia* compounds. Thyme oil was shown to comprise the phenols thymol and its isomer, carvacrol. Human (hBD-1) and murine (mBD-1) β -defensins were examined for activity against *B. cepacia*. In contrast to the salt-sensitive antimicrobial activity observed with *Pseudomonas aeruginosa*, no antimicrobial activity was observed against strains of *B. cepacia*. Elafin, a proteinase inhibitor produced in the human lung was also tested for antibacterial activity against strains of *Staphylococcus aureus*, *P. aeruginosa* and *B. cepacia*. Once again, antimicrobial activity was observed against *S. aureus* and *P. aeruginosa* however, no activity was observed against *B. cepacia*. These observations indicate that members of the *B. cepacia* complex are inherently resistant not only to well-known cationic peptides such as polymyxin, but also to endogenous cationic peptides which were capable of inhibiting a range of pathogens.

This project showed that natural compounds contained within certain plant extracts are bactericidal for strains of the *B. cepacia* complex, whereas most antibiotics and endogenous human cationic peptides lack such activity. At present, the inherent resistance of *B. cepacia* to almost all classic antibiotics including β -lactams, quinolones and aminoglycosides, denies infected patients of effective therapy. The project clearly emphasised the inherent resistance of the *B. cepacia* complex to antibiotics and produced new evidence that this resistance extends to cationic peptides. However, the project also showed that extracts from some plants contain potent antimicrobial activity which includes lethal activity against even the most resistant of the *B. cepacia* epidemic clones. Strategically, it would seem worthwhile to focus further research on the most potent extracts, namely those from thyme oil, and to investigate possible synergistic activity with the most active of the conventional antibiotics.

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DECLARATION

All of the experiments and procedures in this thesis were carried out by the author unless otherwise stated.

CHAPTER 1 INTRODUCTION

1.1 CYSTIC FIBROSIS

Cystic fibrosis (CF) is the most common lethal genetic disease of Caucasians. One in 25 Caucasians of European decent are asymptomatic heterozygous carriers of this autosomal recessive disorder; the incidence of CF in this population is approximately one in 2500 live births. Each year approximately 350 children in the UK are born with CF (Elborn, 1994). CF is less common in other ethnic groups, however cases have been described particularly within the Ashkenazi Jewish population and American blacks (Tsui and Buchwald, 1991). Anderson provided the first comprehensive description of 'cystic fibrosis of the pancreas' in 1938 and named it thus in recognition of the destruction to the pancreatic lesions she observed at autopsy. Of note, many of the patients studied had died as a result of pulmonary infections (Anderson, 1938). In 1953, Di Sant'Agnese and co-workers reported that children with CF had abnormally high levels of sodium and chloride in their sweat (Di Sant'Agnese et al, 1953). This observation lead to development of a diagnostic test for CF based on the measurement of raised sweat electrolytes (Gibson and Cooke, 1959).

CF is a disorder which affects a number of organs including the lungs, gastrointestinal tract, pancreas, liver, vas deferens and sweat glands. It was not until the 1980's that the basic physiological defect in salt transport which affects these organs was described. Poor chloride transport across CF epithelia leads to the production of viscid secretions which cause the major clinical problems associated

with this disease (Quinton, 1983). Viscid bronchial secretions within the lung are thought to lead to poor mucociliary clearance, whereas in the gastrointestinal tract the abnormal secretions can cause blockages termed 'meconium ileus'.

Approximately 90% of CF patients have poor pancreatic function as the viscid secretions block the pancreatic ducts preventing release of pancreatic enzymes (Kubesch et al, 1993). Pancreatic damage can also lead to the development of diabetes in some patients. Other complications of CF include chronic damage to the liver leading to the development of cirrhosis and male infertility due to blocked or absent vas deferens (FitzSimmons, 1993).

1.1.1 Genetic basis of CF

In 1989, the basic genetic defect responsible for reduced ion transport in CF was discovered and the location, sequence and function of the gene responsible were described (Rommens et al, 1989; Riordan et al, 1989; Kerem et al, 1989).

Chromosome walking and jumping techniques along with DNA hybridisation were used to isolate gene sequences from the previously identified CF region on the long arm of chromosome 7 (Tsui et al, 1985), one of these sequences was found to encode the cystic fibrosis gene. This gene sequence was found to encode a protein of 1480 amino acids which has sequence homology with other membrane associated proteins. The gene product was named the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al, 1989). The structure of CFTR was then determined and found to contain two hydrophobic helices which spanned the membrane to form three loops. Each of these membrane spanning domains is linked to a hydrophilic region which contains nucleotide binding folds. In the centre of CFTR there is a

cytoplasmic regulatory domain named the R domain. This region contains sites which can become phosphorylated by protein kinases.

The difference in the sequence of the CFTR gene between CF and unaffected individuals was identified as a three base pair deletion which results in the loss of phenylalanine at position 508 ($\Delta F508$). The position of this crucial amino acid is within the first nucleotide binding fold (Riordan et al, 1989). Subsequent studies indicated that the $\Delta F508$ mutation accounts for approximately 70% of all CF cases (Karem et al, 1989), and is associated with the most severe CF form, including pancreatic insufficiency and meconium ileus. The remaining 30% of cases are made up of over 700 much rarer mutations (Davis et al, 1996).

To confirm that CFTR functions as a chloride ion channel or a regulator for such an ion channel, cDNA for normal CFTR was transfected into a CF pancreatic cell line (Drumm et al, 1990) and into cultured CF airway epithelial cells (Rich et al, 1990). In both studies, the chloride ion channel defect was corrected. Confirmation that CFTR was indeed a chloride ion channel came when highly purified CFTR protein was inserted into artificial cell membranes (planar lipid bilayers) containing no other channel like proteins. When CFTR was incorporated into the bilayer, normal regulated chloride channel activity was observed (Bear et al, 1992).

On present evidence it appears that CFTR functions as a chloride ion channel, which is opened by the hydrolysis of ATP by the nucleotide binding domains (Anderson et al, 1991) and regulated by the phosphorylation of the R domain by protein kinase A (Cheng et al, 1991).

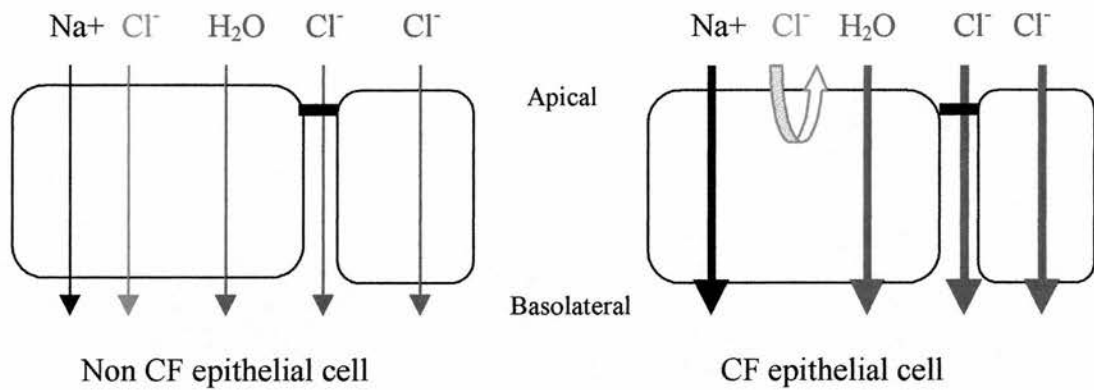
1.1.2 How does defective CFTR cause lung disease?

Two main hypotheses have arisen to provide an explanation of how CFTR dysfunction causes lung disease in CF. Both the low volume hypothesis (Matsui et al, 1998) and the high salt hypothesis (Smith et al 1996; Zabner et al, 1998) focus on disrupted absorption of ions from the airway surface fluid (ASF) of patients with CF.

Low volume hypothesis

In addition to its function as an ion channel, CFTR has now been recognised to function as a regulator of membrane proteins, including other ion channels. Of particular interest is CFTR's function as a regulator of an epithelial sodium channel, ENaC (Stutts et al, 1997). CFTR decreases the amount of time this channel is open, thus, when CFTR function is lost, Na⁺ conductance across the airways is increased. In the low volume theory of Boucher and co-workers, both normal and CF airways are thought to have levels of NaCl similar to those found in plasma. In CF, the mutation of CFTR eliminates the inhibition of ENaC leading to increased sodium absorption. Chloride ions are drawn out of the ASF due to this sodium hyperabsorption, but they travel through the epithelial cells via shunt pathways, as CFTR is not functioning. Water follows the ions back through the cells by osmosis, causing the ASF to become dehydrated. As the volume of ASF is critical to ensure that cilia beat correctly and mucociliary clearance is effective, the low volume of thick mucus left behind in CF airways prevents effective mucociliary clearance. This defective mucociliary clearance leads to an increased incidence of respiratory infections.

Fig 1.1 Low volume hypothesis

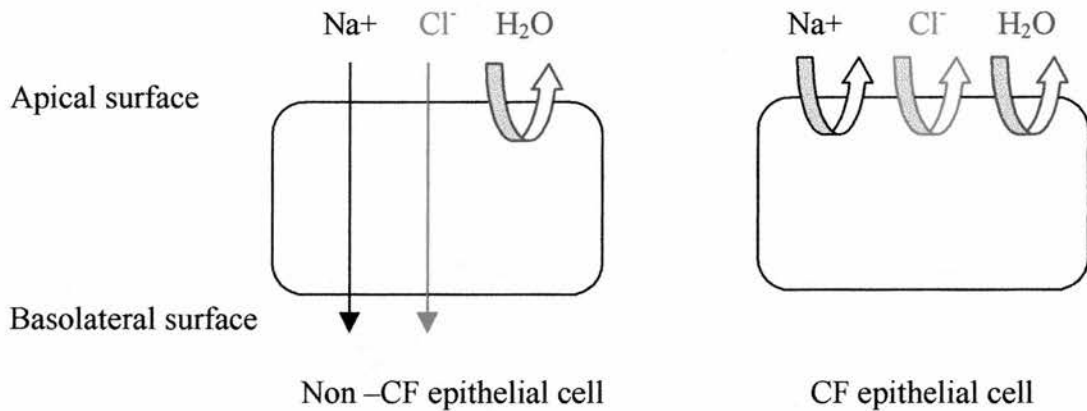


The ENaC channel is shown in black, CFTR in red and alternative pathways for chloride ion transport in green. Na^+ hyperabsorption draws Cl^- and H_2O across epithelial surface which leads to dehydrated ASF.

High salt hypothesis

In this second model, non-CF ASF is considered to have a low, hypotonic salt concentration, which allows salt-sensitive antimicrobial peptides to keep the airways free of bacterial pathogens. In this hypothesis, it is thought that normal airways reabsorb salt, without the consequential flow of water. Sodium is pumped back into the epithelium and chloride follows passively through the CFTR. As Na^+ and Cl^- transport is linked, when CFTR is defective, chloride cannot flow back into the epithelium and only a small quantity of sodium ions can be pumped out of the ASF as a negative charge is produced which prevents further Na^+ movement. High levels of salt accumulate in the ASF which inactivate endogenous antimicrobial peptides (Smith et al, 1996; Zabner et al, 1998).

Fig 1.2 High salt hypothesis



The ENaC channel is shown in black and CFTR in red.

Each of these models provides a plausible theory as to why the CF lung is susceptible to infection. At present, it is technically difficult to measure the salt concentration of ASF accurately as it forms only a very thin film of approximately $30\mu\text{m}$ over the airway surfaces. One study using microanalytical techniques, found that normal ASF had a lower NaCl concentration than CF ASF (Joris et al, 1993). At present, there is not enough evidence to support or disprove either theory. However, there is no doubt that in some way defective CFTR is the cause of an increased susceptibility to lung infections.

1.1.3 Microbial colonisation of the CF lung

Infections of the respiratory tract have long been recognised as a major symptom of CF. In the earliest reports of CF, cultures from bronchial secretions were found to contain *Staphylococcus aureus* in almost every case (Anderson, 1938; 1949). A study of bacterial pathogens isolated from the respiratory tracts of CF patients carried

out between 1950 and 1971 noted that a decline in the number of patients with *S. aureus* infections coincided with an increase in culture of *Pseudomonas aeruginosa*. The introduction of antibiotic therapy had apparently reduced the incidence of staphylococcal infections resulting in an increased incidence of the more antibiotic resistant organism, *P. aeruginosa*. The role of *Haemophilus influenzae* in CF lung disease was also recognised at this time (Mearns et al, 1972).

Advances in treatment of CF, the consequent increase in the life span of patients and improved microbiological procedures has lead to recognition of an increasing range of bacterial and fungal pathogens from CF bronchial secretions. By 1990, it was not unusual to culture *P. aeruginosa* from approximately 60% of CF patients, with an incidence of approximately 20% in children of under one year of age rising to 80% in adult patients. Other *Pseudomonas* species were isolated from around 5% of patients. At this time the *S. aureus* isolation rate was around 28% and the rate of *H. influenzae* isolation 9%. *Candida* and *Aspergillus* species were isolated from approximately 4% and 3% of patients respectively (FitzSimmons, 1993).

Even today a surprisingly restricted spectrum of micro-organisms is responsible for the lung infections of CF patients. *S. aureus* is still the most common pathogen in infancy, often followed by infection with *H. influenzae*. Infections with these organisms can be effectively treated with antibiotics. *P. aeruginosa* is the most common pathogen affecting approximately 60% -90% of adult CF patients in most centres and is the major pathogen responsible for chronic bronchopulmonary infection and progressive lung disease.

P. aeruginosa in CF

By early adulthood, most CF patients have become chronically colonised with *P. aeruginosa*. Within the CF lung, non-mucoid *P. aeruginosa* characteristically converts to a mucoid, non-motile phenotype which is seldom, if ever eradicated (Doggett et al, 1964; Govan and Deretic, 1996). In vivo, mucoid *P. aeruginosa* grows within in complex biofilms made up of alginate, a highly charged bacterial exopolysaccharide released by *P. aeruginosa*, host cellular DNA, and host pulmonary mucins. Within these biofilms, *P. aeruginosa* is less susceptible to antibiotics (Anwar et al, 1992) and to phagocytosis (Govan and Harris, 1986; Simpson et al, 1988).

Several explanations for the increased *P. aeruginosa* colonisation of the CF lung have been proposed. Infection with *P. aeruginosa* is not recognised as a major problem in patients with other mucociliary defects such as ciliary dyskinesia, therefore there may be factors other than poor mucociliary clearance which contribute to the susceptibility of the CF lung to *P. aeruginosa* colonisation. CF respiratory epithelial cells have been shown to bind twice as many *P. aeruginosa* as non-CF cells (Saimen et al, 1990), suggesting that there may be an increase of receptors for *P. aeruginosa* in the CF lung. It has been suggested that abnormalities in CFTR cause alterations in glycosylation which lead to an increased presence of incompletely sialylated glycolipids, such as asialoGM1, a receptor for the major *P. aeruginosa* pilin adhesin (Saiman and Prince, 1993). Rather controversially, it has also been suggested that the first extracellular domain of CFTR also functions as a receptor for *P. aeruginosa* (Pier et al, 1996). In this case, it is postulated that in non-CF lungs, *P. aeruginosa* attaches to CFTR and is removed through normal cellular

desquamation; thus, when CFTR is not present, greater numbers of *P. aeruginosa* can accumulate in the bronchial lumen.

Other factors may also contribute to the susceptibility of CF patients to *P. aeruginosa* colonisation. *P. aeruginosa* lipopolysaccharide (LPS), and cytokines released by inflammatory cells responding to *P. aeruginosa* infection, have been shown to upregulate human mucin genes causing further accumulation of viscid mucin which increases impairment of mucociliary clearance (Dohrman et al, 1998). Inflammatory cytokines and bacterial LPS would normally initiate production of nitric oxide (NO), however levels of NO are reduced in the exhaled air of CF patients, indicating that CF airways are somehow made further susceptible to bacterial infection (Balfour-Lynn et al, 1996).

Accumulated evidence indicates that it is the immunopathological consequences of chronic lung infection with *P. aeruginosa* which ultimately destroy the airways, impair gas exchange and leads to the death of the CF patient. There is evidence to suggest that even CF infants with very mild lung disease have a considerable inflammatory response in their airways with high levels of neutrophils present in their airway secretions (Konstan et al, 1994). It has also been suggested that adherence of *P. aeruginosa* and the subsequent production of the quorum sensing agent, N-acyl homoserine, may induce the production of IL-8 by respiratory epithelial cells which would attract neutrophils into the airways (Moss, 1995). Large numbers of neutrophils in the airways release neutrophil elastase and reactive species, which damages the lung epithelium, causing more neutrophils to be attracted and more cytokines to be released (Doring, 1994).

Ineffective phagocytosis of *P. aeruginosa* growing within protective biofilms would cause an increased release of reactive oxygen intermediates and free radicals from degranulating neutrophils resulting in greater inflammatory damage to the lung. It is this amplification of a frustrated immune response within the lung, caused mainly by chronic *P. aeruginosa* infection which is ultimately the major cause of morbidity and mortality in CF patients (Hutchison and Govan, 1999).

Emerging CF pathogens

Over the last 20 years, there has been an increased recovery of inherently resistant Gram-negative bacteria, including *Stenotrophomonas maltophilia* and *Burkholderia cepacia*, from CF sputum. Early and more intensive antimicrobial treatment of *P. aeruginosa* infections (Denton et al, 1996), coupled with improvements in selective culture media and identification techniques may explain the increased culture rate of *S. maltophilia*. Isolation of *S. maltophilia* from the sputum of a CF patient was first reported in the 1970's (Frederiksen et al, 1995). Information regarding the number of CF patients colonised with *S. maltophilia* has been limited, however a prevalence of as high as 30% has been reported in one clinic (reviewed in Denton, 1997). Transmission of *S. maltophilia* between siblings has been reported, however, the importance of *S. maltophilia* to the morbidity of CF patients is not yet clearly defined.

1.2 *Burkholderia cepacia*

During the 1980's, another bacterial pathogen emerged as a major cause of lung infections in CF patients. The first report of *B. cepacia* colonisation in a CF patient appeared in the 1970's (Ederer and Matsen, 1972), however it was not until the 1980's that the importance of *B. cepacia* as an inherently multi-resistant pathogen of CF patients was confirmed. By 1996, the average culture rate of *B. cepacia* amongst US CF patients was 3.6% (LiPuma, 1998). However, considerable clinic to clinic variation was noted with prevalence rising to 30-40% in clinics where spread between patients has occurred, and reaching as high as 70% in some large clinics (Anonymous 1992; Govan et al, 1996). Although colonisation rate of *B. cepacia* is generally lower than that of *P. aeruginosa*, *B. cepacia* is considered a more dangerous and troublesome problem and causes much anxiety in the CF population.

1.2.1 *B. cepacia* the plant pathogen

B. cepacia was originally described in 1950, as a soil born phytopathogen, capable of causing slimy yellow necrosis of the bulb's outer scales, in the 'yellow globe' variety of onions (Burkholder, 1950). *B. cepacia* could be described as an opportunistic plant pathogen as mechanical damage to the bulb is required before soft rot is initiated. Burkholder suggested that *B. cepacia* did not cause disease of any other plant species. He proposed that this organism should be placed in the *Pseudomonas* genus as it stood at that time, with the species name, *Pseudomonas cepacia* (cepa = onion). However, Burkholder also stated that the strains of *B. cepacia* he analysed possessed certain characteristics that did not fit well with the majority of the plant pathogens

then present in genus *Pseudomonas* (Burkholder, 1950). In 1966, Stanier and co-workers described a group of bacteria recovered from soil, water and clinical environments which they named *Pseudomonas multivorans* (Stanier et al, 1966). Later work established that *P. multivorans* was synonymous with *P. cepacia* (Ballard et al, 1970; Sands et al, 1970). A group of strains isolated from clinical environments originally described as 'eugonic oxidisers group 1' (King, 1964) and subsequently named *Pseudomonas kingii* (Jonsson, 1970), were also synonymized with *P. cepacia* in 1972 (Snell et al, 1972).

1.2.2 The evolving taxonomy of the *B. cepacia* complex

Until 1993 (Anonymous, 1993), *B. cepacia* was officially classified as part of *Pseudomonas* RNA homology group II, one of five subdivisions of this genus proposed by Palleroni and co-workers based on the results of rRNA-DNA hybridisation (Palleroni et al, 1973). A new genus, *Burkholderia*, was subsequently proposed to include seven species belonging to homology group II and with *B. cepacia* as the type species (Yabuuchi et al, 1992). These seven species were able to utilise a greater range of sugars and alcohols than *P. aeruginosa*, the type species of the *Pseudomonas* genus. Subsequent studies have led to the removal of two species to a further new genus *Ralstonia* (Yabuuchi et al, 1995) whilst other 'pseudomonal' species have been added to the *Burkholderia* genus (Urakami et al, 1995; Zhao et al, 1995). The genetic complexity of isolates presumptively identified as *B. cepacia* has led to further taxonomic clarification. *B. cepacia* strains isolated from both clinical and environmental settings, were subdivided into five distinct

genomic species named 'genomovars' on the basis of DNA-DNA and DNA-rRNA hybridisations together with analysis of fatty acid content and whole cell protein profiles (Vandamme et al, 1997). One genomic group, genomovar V, was recognised as the previously described species *Burkholderia vietnamiensis* (Gillis et al, 1995). Another group, genomovar II, was named as a new species, *Burkholderia multivorans* due to distinct biochemical characteristics (Vandamme et al, 1997). Genomovar IV has also recently been described as a new species named *Burkholderia stabilis* in recognition of the limited genomic variability of this species when compared to the diversity of *B. cepacia* strains in general. *B. stabilis* is distinct from other strains of the *B. cepacia* complex by the absence of beta-galactosidase activity and the inability to oxidase sucrose (Vandamme et al, in press) The remaining genomic species are termed 'genomovars' I and III pending identification of distinguishing phenotypic characteristics. Genomovar I contains the type species *B. cepacia* ATCC 25416 and by convention must retain the name *B. cepacia*.

The identification of 'atypical' protein profiles amongst a group of strains isolated from the sputum of CF patients lead to further investigation of their phenotypic and genotypic characteristics. These strains were collectively recognised as a new member of the *B. cepacia* complex and were termed genomovar VI (Coenye et al, b in press). Other strains that were originally tentatively described as *B. cepacia* or *Ralstonia pickettii* like organisms have been included in a new genus *Pandoraea* (Coenye et al, a, in press). At present *Pandoraea* comprises five new species however considering the rapidly evolving taxonomy of the *B. cepacia* complex it would not be surprising if further taxonomic clarification of '*B. cepacia* like' strains is required. At present, the term *B. cepacia* complex is used to refer collectively to

all strains previously classified as *B. cepacia* (Vandamme et al, 1997). For simplicity, unless otherwise stated, the term *B. cepacia* will be used to incorporate all members of the *B. cepacia* complex.

1.2.3 Characteristics of *B. cepacia*

B. cepacia is a Gram-negative, non-spore forming, aerobic bacillus. The species is motile with polar flagella and is typically catalase and oxidase positive. Members have the capacity to produce various non-fluorescent pigments and poly- β -hydroalkanoates can be accumulated as reserve materials. The optimal temperature for growth is 30-35°C (Palleroni, 1984). *B. cepacia* has considerable nutritional diversity, some strains can even utilise penicillin G as a sole carbon and energy source (Beckman and Lessie, 1979). Considerable genetic diversity exists amongst individual strains of *B. cepacia*, even between isolates from a single environmental source. Recombination rates among strains isolated from a single stream were higher than would be expected for binary fission alone (Wise et al, 1995).

Most strains of *B. cepacia* have been reported to possess between two and four chromosomes and can also possess a number of plasmids (Gonzalez and Vidaver, 1979; Lennon and DeCicco, 1991; Cheng and Lessie, 1994; Lessie et al, 1996). Strains of *B. cepacia* have a relatively large genome with a mass ranging from 5 to 9Mb and contains multiple insertion sequences. These numerous insertion sequences can facilitate genomic rearrangements and increase the expression of neighbouring genes (Lessie et al, 1996). *B. cepacia* may also be able to assimilate DNA from

other bacterial species by horizontal transfer which may account for this species' impressive versatility and diversity (Lessie et al, 1996).

Early studies describe *B. cepacia* as ubiquitous in natural environments (Isles et al, 1984; Tablan et al, 1985; Goldman and Klinger, 1986), however this claim has been challenged. Extensive surveillance studies have shown that although *B. cepacia* can be cultured from a number of environmental sources, including water, soil and plants, in particular the plant rhizosphere, isolation rates are relatively low. Rates of isolation from the environment varied from as low as 4% (Fisher et al, 1993; Mortensen et al, 1995) up to 22% (Butler et al, 1995). Surprisingly, even in the homes of CF patients the isolation rates of *B. cepacia* are low (Mortensen et al, 1995).

1.2.4 Bioremediation and biological control

Due to its enormous metabolic versatility, *B. cepacia* has the potential to break down herbicides, pesticides and industrial wastes. Some strains have been shown to degrade highly stable toxic compounds which can contaminate water supplies and soil including trichloroethylene (Krumme et al, 1993) and 2,4,5-trichlorophenoxyacetic acid, the principle herbicide present in agent orange (Kilbane et al, 1983).

B. cepacia also has the ability to repress the growth of many soilborn plant pathogens due to the production of antimicrobial compounds which include pyrrolnitrin (Elander et al, 1968), altericidins (Kirinuki et al, 1977), cepacidine (Lee et al, 1994) and cepacins A and B (Parker et al, 1984). Because of the potential beneficial effects

of this antimicrobial activity, strains of *B. cepacia* are being developed for use as biological control agents to suppress fungal damage in a wide range of commercial crops. *B. cepacia* has been shown to inhibit bacterial wilt disease of tobacco (Aoki et al, 1993) and fungal pathogens of wheat (DeFreitas and Germida, 1991). As seed dressings, *B. cepacia* can control black rot of carrots by *Alternaria radicina* (Chen and Wu, 1999), 'damping off' caused by *Pythium* and *Aphanomyces* root rot of peas (Parke et al, 1991). *B. cepacia* can also colonise the rhizosphere of maize to protect this important crop against soil-borne diseases (Hebber et al, 1992) and suppress fungal spoilage of stored fruit (Janisiewicz and Roitman, 1988).

The economical and ecological benefits to the agricultural industry from the development of *B. cepacia* as a biological control and bioremediation agent are tempered by evidence from medical microbiologists that *B. cepacia* causes life-threatening infections in immunocompromised patients and that most environmentally isolated strains are phenotypically indistinguishable from clinical isolates (Vandamme et al, 1997).

1.2.5 Emergence of *B. cepacia* as human pathogen

Contamination of hospital water supplies, equipment and disinfectant solutions by *B. cepacia* has long been recognised as a cause of opportunistic infections in immunocompromised hosts. Nine cases of *B. cepacia* wound infection were suspected to have arisen from a contaminated 'Savlon' solution (Bassett, 1970). A contaminated chlorhexidine solution was also implicated as the source of an outbreak of *B. cepacia* infections which included wound infections, urinary tract infections and bacteraemias. The chlorhexidine solution had been used to clean infected

wounds, catheters and catheter insertion sites of the patients who became infected (Speller et al, 1971). Contaminated distilled water, used in nebulisers, oxygen humidifier bottles, respirators and croup tents, was also associated with an outbreak of *B. cepacia* infections which occurred among patients undergoing treatment in a hospital intensive care unit (Rapkin et al, 1976). Van Laer et al, (1988) reported eight cases of septicaemia due to *B. cepacia* affecting patients on a cardiology ward over a three-day period. The infections were thought to have arisen from heparin injections, where the heparin had been diluted with a dextrose solution contaminated with *B. cepacia*. In another study, a batch of contaminated human serum albumin was traced as the source of an outbreak of *B. cepacia* infections which affected 15 patients in five hospitals (Steere et al, 1977). Other sporadic nosocomial *B. cepacia* infections have been reported including cases of endocarditis observed in heroin addicts. (Noriega et al, 1975).

B. cepacia infection of immunocompetent hosts has also been documented.

B. cepacia was shown to be the cause of macerated, hyperkeratotic foot lesions in healthy Marines training in swampy conditions (Taplin, 1971). In addition, *B. cepacia* was found to be the cause of community acquired pneumonia in an immunocompetent host (Pujol et al, 1992). Most recently, ear drops contaminated with *B. cepacia* were implicated as the cause of multiple brain abscesses secondary to otitis media, in an otherwise healthy offshore oil worker (Hobson et al, 1995). Certain inherited genetic disorders may also increase susceptibility to *B. cepacia* colonisation and infection. In particular, patients with chronic granulomatous disease (CGD) appear particularly vulnerable to serious lower respiratory tract infections with *B. cepacia* (O'Neil et al, 1986). CGD is a neutrophil disorder in

which cells do not produce oxidative reactive intermediates such as hydrogen peroxide. The association of *B. cepacia* with life-threatening infections in patients with CGD is thought to be due to resistance of this organism to nonoxidative killing by neutrophils (Speert et al, 1994).

1.3 *B. cepacia* in CF

Although the first report of *B. cepacia* colonisation of a CF patient appeared in 1972 (Ederer and Matsen, 1972), it was not until the late 1970s that *B. cepacia* was recovered with increasing frequency from individuals with CF. In 1980, a case of *B. cepacia* pneumonia and septicaemia in a 17-year-old CF patient was described (Rosenstein and Hall, 1980). Subsequently, the seminal report by Isles and co-workers (Isles et al, 1984) highlighted the increased incidence of *B. cepacia* colonisation in Western Canada during the previous decade and described the variable clinical outcomes observed amongst *B. cepacia* colonised patients. Once a patient is colonised with *B. cepacia* one of three clinical sequelae can occur. *B. cepacia* can be carried chronically and asymptotically either alone or in combination with *P. aeruginosa*. Alternatively, the patient's lung function may progressively deteriorate over many months accompanied by fever and weight loss, in much the same pattern as infection by *P. aeruginosa*. The third and most severe outcome of *B. cepacia* colonisation is 'cepacia syndrome' a rapidly fatal and necrotising pneumonia which can be accompanied by septicaemia. Of particular concern, it was noted that cepacia syndrome could occur unexpectedly in patients who have relatively 'mild' CF disease.

B. cepacia colonisation of CF patients continues to provoke major concern within the CF community for a number of reasons. Colonisation is associated with poorer clinical outcome, not only for the 20-30% of colonised patients who experience the rapid decline associated with cepacia syndrome, but for most patients who become colonised with this organism. Furthermore, evidence from national CF databases shows that colonisation reduces life expectancy by 50% (Corey, 1999). *B. cepacia* is also difficult to treat because of its innate resistance to antibiotics. In addition, evidence strongly suggests that direct patient-to-patient transmission of *B. cepacia* can occur which may account for the high colonisation rates recorded in some treatment centres.

1.3.1 Transmission of *B. cepacia*

Escalation in the incidence of *B. cepacia* colonisation during the 1980's among CF patients may have partly been due to the development of selective media for the isolation and growth of this organism and improved identification techniques (Gilligan et al, 1985; Tablan et al, 1987). However, it is likely that other factors were also responsible. Early studies highlighted apparent risk factors for *B. cepacia* colonisation which included having a colonised sibling or having spent periods in hospital (Tablan et al, 1985; 1987). Clustering of cases was also observed in some CF centres leading to speculation that specific hospital practices or person-to person spread may be responsible for increased prevalence (Goldman and Klinger, 1986). Implementation of stringent infection control procedures, including physical separation of *B. cepacia* colonised patients from those who were not colonised,

dramatically reduced the incidence of colonisation at one centre (Thomassen et al, 1986).

Accumulating evidence in the late 1980's continued to suggest a role for person to person transmission in new cases of *B. cepacia* colonisation. However, it was not until 1988 that use of a genomic fingerprinting technique known as ribotyping confirmed that the majority of *B. cepacia* colonised patients at each of three CF treatment centres were colonised with the same strain although the epidemic strain in each of the three treatment centres was different (LiPuma et al, 1988). Moreover, ribotyping was also used to provide further scientific evidence for person to person transmission between two CF patients who were attending a summer camp. One CF patient became colonised with the same strain of *B. cepacia* as another CF camper after only six days of social contact. However, the possibility of that transmission occurred via contaminated environmental sources was not examined (LiPuma et al, 1990). *B. cepacia* colonised patients can contaminate the environment thus the potential for transmission via contaminated environmental sources does exist (Nelson et al, 1991).

Subsequently, several other studies provided further evidence of transmission of *B. cepacia* between CF patients. Pegues and co-workers sequentially examined the sputum or throat cultures of patients attending one of three CF summer camps for *B. cepacia*, upon arrival, each week during the camp, at the end of camp and 14 and 30 days after the camp. Ribotypes of the *B. cepacia* isolates from patients with sputum conversion were compared with isolates from other campers and those from environmental sources. All 11 campers who became colonised with *B. cepacia* during the study period had become colonised with strains possessing identical or

very similar ribotypes to each other and to those isolated from previously colonised campers. Examination of environmental sources yielded only one isolate of *B. cepacia* which had a ribotype unique from the patients attending the camp where it was isolated. (Pegues et al, 1994).

1.3.2 Epidemic *B. cepacia* strains

By the early 1990s, it was recognised that certain strains of *B. cepacia* appear to be highly transmissible. One such 'epidemic' strain was recognised as the cause of 33 of the 60 cases of *B. cepacia* colonisation among patients attending Edinburgh and Manchester CF clinics between 1986 and 1992 (Govan et al, 1993). Genotyping and phenotyping techniques along with a detailed study of contacts between cases provided compelling evidence that this epidemic strain had been spread between patients through social contact, both within and between each of the centres by attendance of patients from both these clinics at a weekend camp for CF patients. Details of both social and hospital contacts between patients provided insight into the level of contact required for transmission of this epidemic strain. Regular social contact at fitness classes and social kissing appeared to be all that was necessary for transmission of this strain. Within the Edinburgh clinic, 50% of *B. cepacia* colonised CF patients who died during this study period were colonised with this epidemic strain. However, clinical outcome following colonisation cannot be predicted and there was no deterioration in the health of some individuals. Poor lung condition before colonisation tended to be associated with poor survival, however this did not appear to be associated with an increased risk of colonisation. The

'Edinburgh epidemic strain' was later recognised as the same strain that was responsible for an ongoing epidemic in the Toronto region of Canada. This strain later to be known as the notorious Edinburgh / Toronto ET12 lineage (Govan et al, 1996), may have been spread between patients from these two continents while they were attending a summer camp (Johnson et al, 1994).

1.3.3 Segregation of *B. cepacia* colonised patients

The risk to the CF community of transmission of *B. cepacia* led to UK guidelines recommending that individuals colonised with *B. cepacia* should avoid sleeping, exercising and performing physiotherapy in the same room as non-colonised individuals and that kissing and the sharing of physiotherapy equipment and eating utensils should be avoided. The risks involved in attending conferences and CF summer camps where *B. cepacia* colonised individuals may be present were also highlighted (Govan et al, 1992). Similar guidelines were also produced in Europe, Canada and in the USA. It is not an exaggeration to say that the implication of these strict infection control policies has led to considerable distress and debate in the CF community. Many of the social activities including summer camps which built friendships and community spirit amongst CF patients have been discontinued and *B. cepacia* colonised individuals are left feeling like outcasts (Anonymous, 1992). In a further twist, recent case studies including a report of a previously healthy mother who acquired *B. cepacia* from her CF children and developed severe bronchiectasis (Ledson et al, 1998a) and a report of fatal cross infections with the epidemic strain between patients already colonised with other *B. cepacia* strains

(Ledson et al, 1998b) suggests that even more draconian infection control procedures may be necessary.

Accumulating evidence suggests that not all strains of *B. cepacia* are equally transmissible nor are all members of the *B. cepacia* complex associated with virulence (Vandamme et al, 1997; LiPuma, 1999; Hutchison and Govan, 1999).

With greater understanding of the pathogenicity of *B. cepacia* and the host factors that may be involved in the development of the more severe outcomes of *B. cepacia* colonisation, it may be that in the future, segregation could be relaxed for patients colonised with non-transmissible strains. However, although unpopular, reliable surveillance and segregation of new cases is essential to reduce spread of *B. cepacia* colonisation (Govan, 2000).

1.4. Virulence determinants of *B cepacia*

In contrast to an increased understanding of *B. cepacia* epidemiology, the pathogenicity of *B. cepacia* is relatively unclear. Several putative virulence factors of *B. cepacia* have been identified, however their relevance to colonisation and damage of the CF lung has not yet been fully described. A greater understanding of the mechanisms involved in colonisation of the CF lung and the more severe pulmonary deterioration which occurs in some patients could potentially lead to effective treatment for *B. cepacia* infections.

1.4.1 Adherence

Adherence of bacterial fimbriae or pili to host epithelial and mucosal surfaces is often the first step in the establishment of infection by bacterial pathogens. An adhesin, which facilitates attachment of *B. cepacia* to mucin and buccal epithelial cells, has been described (Sajjan and Forstner, 1992; 1993). The 22kDa protein shown to be located on pili is present in only certain *B. cepacia* strains.

Subsequently, a 17kDa pilin subunit was purified from one the isolates previously shown to express the 22kDa adhesin. Pilin subunits are thought to interact with other gene products to permit the assembly of fimbriae. This 17kDa pilin subunit assembles into large peritrichous fimbriae, with which the 22kDa mucin binding adhesin is associated (Sajjan et al, 1995). These pili, named cable or cbl type II pili, constitute one of five different classes of fimbriae expressed on the surface of clonally diverse strains of *B. cepacia* (Goldstein et al, 1995). The cable pilus was first identified in the highly transmissible ET12 strain of *B. cepacia*, which lead to speculation that the cable pilus phenotype may identify other highly transmissible *B. cepacia* strains (Sun et al, 1995). However, the cable pilus has not been identified in other epidemic strains (Mahenthiralingam et al, 1997). Four other morphologically distinct classes of peritrichous fimbriae in *B. cepacia* have been identified by electron microscopy. Type III filamentous (Fil) pili are expressed mainly by CF isolates of *B. cepacia* where as type IV spine (Spn) pili are mainly expressed by non CF clinical isolates and type V spike (Spk) by environmental isolates. Cable, filamentous and spike pili are expressed on the cell surface in association with the final pilin class, type I mesh (Msh) pili (Goldstein et al, 1995).

A novel genomic marker termed '*B. cepacia* epidemic strain marker' (BCESM) has been identified in seven epidemic strains of *B. cepacia*. However, this marker was found to be absent from non-epidemic strains isolated from CF patients and rare among strains from the natural environment. The absence of BCESM for non-transmissible strains suggests that it may provide a useful marker for transmissibility. The role of the protein encoded by the 1.4 kb BCESM DNA has not yet been identified (Mahenthiralingam et al, 1997).

1.4.2 Extracellular virulence factors

Strains of *B. cepacia* have been shown to produce a number of extracellular products which may play a role in the pathogenicity of this species. A number of factors such as proteases, lipases, haemolysins and siderophores have been closely examined, whilst other characteristics have merely been identified as more common in clinical than in environmental strains. Isolates from CF patients more frequently produce catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginase and trypsin; reduce nitrate to nitrite; hydrolyse urea and xanthine and hydrolyse bovine red blood cells (Gessner and Mortensen, 1990). However, the function these characteristics may have in the pathogenesis of *B. cepacia* infections is unknown.

Protease

B. cepacia has been shown to secrete at least two proteases. A 32kDa protease with antigenic similarity to *P. aeruginosa* elastase has been shown to cleave gelatin, hide powder and collagen but not the human immunoglobulins IgG, IgM, IgA or secretory

IgA. Purified protease caused bronchopneumonia, characterised by polymorphonuclear cell infiltration and proteinaceous exudation, within rat lungs. Immunisation of rats with this protease elicited an immune response, however, it was not protective against subsequent lung infection with *B. cepacia* (McKevitt et al, 1989). A second, 40kDa protease with much lower activity is also produced by some strains of *B. cepacia* (Kooi et al, 1996).

Lipase

Several reports have described lipolytic activity in *B. cepacia* (Carson et al, 1973) (McKevitt and Woods, 1984; Lonon et al, 1988; Straus et al, 1992). The majority of strains tested had some lipolytic activity, however, activity appeared to be strain dependent. The toxin purified by Lonon and co-workers was not cytotoxic to HeLa cells or when injected intravenously into mice. However, this lipase was subsequently shown to significantly reduce phagocytosis by rat pulmonary alveolar macrophages both when macrophages were preincubated with lipase and when phagocytosis occurred in the presence of lipase. Morphological changes were observed in macrophages incubated with this lipase (Straus et al, 1992).

Phospholipase C has been associated with lung cytopathology due to its ability to cleave phosphatidylcholine, a major lung surfactant. Reports have shown that phospholipase C (PLC) activity amongst strains of *B. cepacia* can vary from 40% (Vasil et al, 1990) to as high as 70% (Nakazawa et al, 1987) and is associated with haemolytic activity in some strains of *B. cepacia*. However, unlike *P. aeruginosa* the level of PLC activity of *B. cepacia* does not correlate with haemolytic activity (Vasil et al, 1990).

Haemolysin

Several haemolysins have been isolated from strains of *B. cepacia*. The haemolytic activity observed by Vasil and co-workers (1990) was always associated with some level of PLC activity, and appeared to require the expression of two closely linked genes. Another study characterised a heat sensitive haemolysin from *B. cepacia* (Nakazawa et al, 1987). In this study, all haemolytic strains were found to possess PLC activity, however only 4% of strains in this study were haemolytic. Cepalysin, a compound displaying haemolytic and antifungal activity, isolated from a Japanese clinical isolate of *B. cepacia* and has been shown to form pores of between 20 and 30nm in red blood cells (Abe and Nakazawa, 1994). More recently, another haemolysin has been isolated, on this occasion from the highly transmissible ET12 strain of *B. cepacia* (Hutchison et al, 1998a). This lipopeptide toxin produced pores in human red blood cells of less than 20nm. When neutrophils were exposed to low levels of this toxin, DNA degradation typical of apoptotic programmed cell death was observed. High levels of haemolysin resulted in the release of both cathepsin G and elastase from human neutrophils. It is tempting to hypothesise that this toxin may contribute to the severe inflammatory response often observed in patients colonised with this bacterium.

Siderophores

Bacteria that produce siderophores can compete with host iron binding proteins which improves the ability of potential pathogens to establish and maintain infection. To date, *B. cepacia* strains have been shown to produce three siderophores,

pyochelin, cepabactin and azurechelin. 51% of the clinical isolates studied by Sokol (1986) were pyochelin negative, thus this siderophore may not be important for the establishment of infection in CF patients. However, increased morbidity and mortality was reported in CF patients colonised with pyochelin producing strains (Sokol, 1986). Cepabactin and pyochelin are both produced by the type strain of *B. cepacia* (ATCC 25416), an environmental isolate (Meyer et al, 1989). Cepabactin is reported to strongly chelate iron and to facilitate iron translocation. Azurechelin was found to be produced by 88% of CF respiratory isolates of *B. cepacia* and thus may have a stronger association with virulence than pyochelin (Sokol et al, 1992). The association of siderophores with virulence of *B. cepacia* however, requires further investigation.

Exopolysaccharide production

Unlike *P. aeruginosa*, the majority of *B. cepacia* strains do not appear capable of producing alginate-like exopolysaccharides. A non-alginate heteropolysaccharide has been reported in some strains of *B. cepacia*, however production is not thought to correlate with colonisation or virulence of *B. cepacia* (Nelson et al, 1994). In contrast to *P. aeruginosa*, isolation of mucoid forms of *B. cepacia* from CF patients is rare (Govan and Deretic, 1996).

1.4.3 Inflammatory damage

B. cepacia colonisation has been shown to produce a pronounced humoral response. Serum IgG and sputum IgA antibodies directed towards the core of *B. cepacia* LPS

have been identified in the sera of *B. cepacia*-colonised CF patients. The presence of these antibodies did not appear to prevent bacterial colonisation and no cross reactivity was observed with *P. aeruginosa* LPS (Nelson et al, 1993). However, IgG antibodies to outer membrane proteins of *P. aeruginosa* have been reported to crossreact with surface antigens of *B. cepacia* (Arnoff et al, 1991). As *P. aeruginosa* often colonises the CF lung before *B. cepacia* it was concluded that antibodies to outer membrane proteins did not inhibit *B. cepacia* colonisation.

Interestingly, the LPS of *B. cepacia* appears to be capable of causing considerably more immunological damage to lung tissue than *P. aeruginosa* LPS. On a weight-for-weight basis LPS preparations from both clinical and environmental isolates of *B. cepacia* have been shown to induce the production of approximately nine times as much TNF- α as *P. aeruginosa* LPS (Shaw et al, 1995). Neutrophil activation by *B. cepacia* LPS has also been described. Increased expression of complement receptor 3, important for neutrophil transmigration into alveolar spaces, and priming of neutrophil respiratory burst activity, was observed for both clinical and environmental isolates of *B. cepacia* (Hughes et al, 1997). LPS from the ET12 strain of *B. cepacia* has also been shown to upregulate the production of inducible nitric oxide synthase and inflammatory cytokine mRNA in CF and non-CF leukocytes (Hutchison et al, 1998b).

An extracellular factor distinct from LPS has also been shown to induce a cytokine response. Cell free supernatants from clinical and environmental isolates of *B. cepacia* were found to stimulate the release of IL-8 from epithelial cells and human monocytes (Palfreyman et al, 1997). Studies have also shown that levels of neutrophil elastase and C-reactive protein are raised during exacerbations with

B. cepacia (Elborn et al, 1994), and that *B. cepacia* stimulates a pronounced inflammatory response in CF mouse model (Davidson et al, 1995). The susceptibility of patients with CGD to infections with *B. cepacia* is thought to be due to resistance of this organism to nonoxidative killing by human neutrophils (Speert et al, 1994). It is not yet known if this feature contributes to survival of *B. cepacia* in the CF lung.

In conclusion, although no definite role has been described for many of the other virulence factors described to date, increasing evidence suggests that endotoxin activation and subsequent acute inflammatory response are important factors in the acute pulmonary deterioration often associated with *B. cepacia* infection.

1.4.4 Antimicrobial resistance

Most *B. cepacia* possess intrinsic resistance to many antibiotics and antibacterial agents (Lewin et al, 1993), exemplified by an ability to use penicillin G as a carbon source (Beckman and Lessie, 1979). Even when sensitivity to antibiotics is demonstrated in vitro, antibiotic treatment is often ineffective thus depriving colonised patients from effective antibiotic therapy. Several mechanisms have been described to account for the resistance of *B. cepacia* to a variety of antimicrobial agents.

β-lactam resistance

Resistance of *B. cepacia* to β-lactam antibiotics is thought to be partly due to low permeability of the outer membrane and partly due to inducible β-lactamases. The

outer permeability of *B. cepacia* to the β -lactam nitrocefin was reported to be ten times less than that of *E. coli* and similar to *P. aeruginosa* (Parr et al, 1987).

Unusually small porins with low single channel conductance, are present in the *B. cepacia* outer membrane which may account for the low permeability of the membrane.

Decreased expression of a 36kDa outer membrane protein and loss of a 27kDa protein has also been associated with decreased permeability and β -lactam resistance in isolates of *B. cepacia* (Arnoff, 1988). The 27kDa outer membrane protein may be a major porin or major component of a porin complex of *B. cepacia* (Arnoff, 1988).

Beckman and Lessie first identified a highly inducible β -lactamase activity in *B. cepacia*. Strains which had this enzyme could also utilise penicillin G their sole source of carbon and energy (Beckman and Lessie, 1979). Two separate β -lactamases have been identified in *B. cepacia* ATCC 17616. The penicillinase originally described by Beckman and Lessie is responsible for about 80% of the β -lactamase activity of this strain. A second β -lactamase with mainly cephalosporinase activity accounts for the rest of the activity (Hirai et al, 1980; Prince et al, 1988).

The penicillinase described by Beckman and Lessie (1979) was subsequently sequenced and recognised to belong to the molecular class A β -lactamases. This chromosomal β -lactamase, *penA*, is regulated by the *penR* gene and has high basal and hyperinducible activity (Trepanier et al, 1997). The β -lactamases produced by *B. cepacia* strains also exhibit carbapenamase activity (Simpson et al, 1993; Iaconis et al, 1993; Trepanier et al, 1997). Resistance to imipenem is thought to be due to reduced permeability brought about by loss or decreased expression of a 36kDa outer

membrane protein and to the production of carbapenemases. Meropenem was found to be more stable to β -lactamases than imipenem (Iaconis et al, 1994). The *penA* encoded penicillinase isolated from ATCC17616 also has activity against imipenem (Trepainer et al, 1997).

Aminoglycoside resistance

B. cepacia strains are invariably resistant to aminoglycoside antibiotics. This resistance has been attributed to low outer membrane permeability (Parr et al, 1987). However, resistance is not considered to be due to small porin size or to decreased porin expression, but rather to properties of bacterial LPS. Aminoglycosides and polypeptide antibiotics such as colistin and polymyxin B interact with the outer membrane of Gram-negative bacteria as the first step of their antimicrobial activity. The antibiotics displace divalent cations that crossbridge and provide stability between LPS molecules. Cracks are then formed in the outer membrane allowing for greater uptake of these antibiotics. Polycationic antibiotics can thus reach their final target, the negatively charged cytoplasmic membrane in which they form channels, destroying membrane integrity and causing death of the bacterial cell (Moore and Handcock, 1986).

It has been suggested that in this 'self promoted uptake' system, aminoglycoside antibiotics cannot breach the outer membrane of *B. cepacia* as divalent cations are not responsible for crosslinking between LPS molecules. High levels of 4-amino-4-deoxyarabinose, present in the LPS of *B. cepacia*, may confer polymyxin resistance as its presence makes the binding of divalent cations unnecessary for outer membrane stability. To provide stability, positively charged arabinose forms ion

pairs with negatively charged phosphate molecules present in adjacent LPS molecules (Cox and Wilkinson, 1991; Vaara, 1992). The overall negative charge of *B. cepacia* LPS is explained by low levels of KDO and phosphate compared to other Gram-negative bacteria. In conclusion, the capacity of the outer membrane of *B. cepacia* to bind cations is low (Cox and Wilkinson, 1991).

Resistance of B. cepacia to other antibiotics

Plasmid encoded tetracycline and ampicillin resistance has been reported in a single strain of *B. cepacia* (Williams et al, 1979). However, surprisingly few antimicrobial resistance genes have been detected on *B. cepacia* plasmids.

Resistance of *B. cepacia* to several antibiotics has been attributed to low outer membrane permeability. Impermeability has been reported to account for the high level of resistance of *B. cepacia* to chloramphenicol, trimethoprim and ciprofloxacin, although no difference in LPS or outer membrane proteins was detected (Burns, 1989). However, resistance of *B. cepacia* to trimethoprim, ciprofloxacin and chloramphenicol has recently been associated with the expression of an antibiotic efflux pump. Subcloning of a 3.4kb fragment of *B. cepacia* DNA revealed one complete and one partial open reading frame which are homologous with two of the three components of an efflux operon of *P. aeruginosa* (*mexA-mexB-oprM*) (Burns et al, 1996b). Thus, resistance to several antibiotics by *B. cepacia* may not solely be due to reduced permeability; active efflux may be an important factor.

Resistance in the CF lung

B. cepacia is able to invade respiratory epithelial cells. Intracellularity would enable strains that appear sensitive to antibiotics in vitro, to survive within the CF lung despite antibiotic therapy. This invasive potential would also explain to some extent why this CF pathogen and not *P. aeruginosa* can cause bacteraemic infections (Burns et al, 1996a).

Conditions which exist in the CF lung may also affect the susceptibility of *B. cepacia* to antibiotics in vivo. Susceptibility of *B. cepacia* strains to β -lactam antibiotics is reduced when incubated in an atmosphere of 5% carbon dioxide. This factor may be relevant in vivo since patients with advanced CF may have high alveolar carbon dioxide concentrations (Corkill et al, 1994). Growth of *B. cepacia* in nutrient depleted conditions has also been shown to increase resistance to antimicrobial agents. Thus, nutrient depletion in vivo may cause strains to become more resistant to antibiotics (Cozens and Brown, 1983). Differences in oxygen availability in the CF lung due to accumulation of mucin and bacterial exopolysaccharide may also have an effect on the susceptibility of strains of *B. cepacia* to antibiotics in vivo.

Susceptibility of *B. cepacia* to ciprofloxacin and tobramycin increased in conditions of oxygen depletion (McKenney and Allison, 1997). Bacteria may experience oxygen depletion when trapped within layers of alginate, mucin and other host debris present in the CF lung. However, antibiotic activity in the lung may also be reduced, if penetration into the glycocalyx-like biofilm surrounding *B. cepacia* is impaired.

1.5 Plant extracts as therapeutic agents

For centuries, plant extracts have been used to treat a wide range of medical conditions. The World Health Organisation estimates that 80% of the world's population use herbal medicines for some aspect of primary health care. By 1980, the United Nations had identified 400 plant derived medicines in commercial use (Jones, 1996). It has been calculated that approximately, one in four prescription drugs dispensed by local chemists in the USA contain at least one ingredient derived from plants. Medicines derived from plants include analgesics, anaesthetics, oral contraceptives, heart drugs and anti-cancer treatments (Lewington, 1990).

Salicylic acid, a compound isolated from meadowsweet (*Filipendula ulmaria*) and related to salicin, a well-known painkilling compound from willow trees (*Salix alba*), was modified by German chemists to produce aspirin (acetyl salicylic acid) (Jones, 1996). Two other more potent pain relieving compounds, morphine and codeine are derived from the opium poppy (*Papaver somniferum*). Morphine was chemically modified to produce the even more effective painkiller, heroin.

Digitalis extracted from the foxglove (*Digitalis* spp.) comprises a mixture of glycosides which include digitoxin, digoxin and lanatoside c. These chemicals have beneficial effects on the cardiovascular system including regulating heartbeat, stimulating more forceful contractions and increasing the muscular activity of the heart (Lewington, 1990).

The muscle relaxant, tubocurarine, often used in abdominal surgery, comes from the stem of *Chondrodendron tomentosum*, which is used by South American Indians as a source of arrow poison. Another well known relaxant, atropine from deadly nightshade (*Atropa belladonna*) is used to dilate the pupil of the eye for diagnostic

purposes and as a treatment for stomach and duodenal ulcers. Diosgenin, a steroidal sapogenin that occurs naturally in yam species, is utilised for the large scale production of male and female hormones required for the manufacture of oral contraceptives (Lewington, 1990). Taxol, a drug extracted from the pacific yew tree (*Taxus baccata*) has been developed as an anti-cancer treatment.

A number of studies have reported the antimicrobial activity of plant extracts. Since even the most potent antipseudomonal antibiotics are not effective in eradicating *B. cepacia* from the CF lung, one of the major aims of this thesis was to focus on plants, in particular garlic (*Allium sativum*), thyme (*Thymus vulgaris*), as potential sources of novel antimicrobial agents against this untreatable opportunistic pathogen.

1.5.1 History of the medicinal use of garlic

Extracts of garlic (*Allium sativum*), a member of the Liliaceae family, have been used therapeutically for several thousand years. The Sumarians produced some of the first written accounts of the medicinal properties of garlic extracts around 2600 - 2100 BC. The Egyptians also recorded their use of garlic extracts as medicines in the Codex Ebers (1550 BC), discovered by the German Egyptologist Georg Ebers in 1872. This document contains 800 therapeutic formulations, 22 of which mention garlic (Block, 1985).

Dioscorides, a Greek who accompanied the Roman troops in Asia as an official physician in the first century AD, wrote the first detailed description of the therapeutic uses of garlic (Stoll and Seebeck, 1951). Included in these records were descriptions of the use of garlic as a treatment for skin rashes, parasitic infections of

the skin, leprosy and as a remedy for snake bites and the bites of mad dogs was recorded. Textbooks of Sanskrit medicine, known as 'Ayurveda' (Science of Life) written around 500AD contain information about the three legendary Indian physicians Charaka, Susruta and Vagbhata who used garlic, which they called 'mahushuea', as a remedy for skin and abdominal diseases.

In the early 18th century, four condemned criminals, recruited to bury victims of plague in Marseilles supposedly drank a concoction of garlic juice and wine to 'protect' them against the disease. This mixture is known as 'vinaigre des quatre voleures' and can still be purchased in France.

Louis Pasteur reported the antibacterial properties of garlic in 1858. Later, Albert Schweitzer (1875-1965), is stated to have treated amoebic dysentery with garlic. Garlic was also used during both world wars as an antiseptic to prevent gangrene (Block, 1985).

1.5.2 Discovery of allicin

The scientific basis for the medicinal use of garlic was not established until 1930, when it was shown that garlic could inhibit the growth of *E. coli* (Lehmann, 1930). In 1944, Cavallito and Bailey demonstrated that a relatively unstable, colourless oil obtained from the steam distillation of ethanolic extracts of garlic retained antimicrobial activity at concentrations as low as 1:125000. They named the active agent they had discovered 'allicin' (allyl 2 propene thiosulphinate). This sulphur containing compound was also shown to have considerable activity against both Gram-positive and Gram-negative bacteria. Stoll and Seebeck later elucidated the pathway by which allicin is formed in garlic (Stoll and Seebeck, 1951). Briefly,

allicin is not present within intact garlic bulbs. However, the garlic bulbs contain the precursor compound, 'alliin' ((+)-S-allyl-L-cysteine sulphoxide). This compound is hydrolysed to allicin by the plant enzyme, alliinase, which comes into contact with alliin when a bulb is cut or crushed. In 1956, Willis reported, that allicin is an inhibitor of sulphydryl enzymes suggesting that the antibacterial properties of allicin may be due to interference with SH-groups (Willis, 1956).

1.5.3 The sulphur chemistry of garlic

Garlic has a particularly high content of sulphur compounds with 1.1 to 3.5% of the fresh weight of garlic comprising such compounds (Lawson, 1995). Wertheim (1844), was first to demonstrate that the compounds that give garlic its distinctive smell are organosulphur compounds but he incorrectly proposed that these compounds have the same basic formula $C_6H_{10}S$. It was almost 50 years later, that Semmler correctly determined the basic formula as C_3H_5S and identified the specific compounds. The oil obtained from steam distillation of garlic bulbs was found to consist mainly of diallyl disulphide (60%), diallyl trisulphide (20%) and diallyl tetrasulphide (10%) (Semmler, 1892). It was not until 1944, that Cavallito and Bailey discovered the major antibacterial component of crushed garlic, allyl-2-propene thiosulphinat, which they named allicin. They observed that the antimicrobial activity of allicin was reasonably stable at 4°C but rapidly lost activity on heating. Furthermore, activity was immediately lost upon addition of alkali whereas dilute acids had no effect (Cavallito and Bailey, 1944a; b; 1945).

Alliin can be synthesised by the mild oxidation of diallyl disulphide (Stoll and Seebeck, 1951). Experiments carried out with a dried, low temperature, acetone extract of garlic showed that no diallyl sulphides are present in intact garlic bulbs and that alliin is not formed when organic solvents are present or in the absence of water (Cavallito et al, 1945). It was concluded that alliin must be formed from a parent compound by the action of an enzyme which is inactivated by ethanol and that the diallyl sulphides present in the oil of steam distilled garlic are derived from alliin. The precursor of alliin was found to be an oxygenated sulphur amino acid named alliin and the enzyme that converts this substrate to alliin was identified and named alliinase (Stoll and Seebeck, 1951). Alliin is also formed from a parent compound, γ -glutamyl-S-allylcysteine, the second most abundant of nine different γ - glutamyl peptides present in garlic (Lawson et al, 1991b).

Alliin and alliinase are located in different cell types within garlic bulbs a fact that might be expected from the absence of thiosulphinates production until cloves are cut or crushed. Alliinase is located in the bundle sheath cells located around the phloem (Ellmore and Feldberg, 1994) whereas alliin is found in the more abundant storage mesophyll cells of the plant (Lawson, 1995). Interestingly, this is a different situation from that which exists in onion bulbs where alliinase and the cysteine sulfoxides are located in different compartments of the same cell (Lancaster and Collin, 1981).

Alliin is the principle thiosulphinate compound produced when a garlic bulb is cut or crushed. The enzyme, alliinase, is also responsible for catabolising reactions of other cysteine sulfoxides including methiin and isoalliin and for production of other thiosulphinates including two allyl methyl thiosulphinates, two allyl trans-1 propenyl

thiosulphinates, two methyl trans-1-propenyl thiosulphinates and methyl methanethiosulphinate (Lawson et al, 1991c).

Not surprisingly, garlic's well known and long established medicinal properties have led to a 'garlic industry' producing numerous commercial preparations. These products include garlic powders, steam distilled garlic oils, oils of macerated garlic and minced garlic aged in ethanol. It should be noted that high performance liquid chromatography (HPLC) analysis has shown that only aqueous garlic extracts and dried garlic powders contain thiosulphinates (Lawson et al, 1991a).

HPLC is the most reliable method to identify organosulphur compounds present in garlic preparations. The apparatus can be operated at ambient room temperatures, and thus can measure allicin and other thiosulphinates at temperatures which avoid the destabilising injection port and column temperatures of gas chromatography.

Studies have shown that C₁₈ HPLC and Si HPLC used in association with external standards produce reliable qualitative and semiquantative measure of the compounds present in garlic preparations (Jansen et al, 1987; Lawson et al, 1991a; Lawson et al, 1991c). Si HPLC with 2-propanol / hexane gradients produces the best resolution of isomers present in thiosulphinate mixtures, however poorer separation of these isomers is also achievable with C₁₈ HPLC (Block et al, 1992). A spectrophotometric method for the quantification of allicin and other thiosulphinates, developed by Han and co-workers, is based on a reaction reported in 1944 by Cavallito and co-workers. One molecule of allicin reacts with two molecules of cysteine to form S-allylmercaptocysteine. The decrease in the amount of cysteine present can be measured spectrophotometrically using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Han et al, 1995).

Allicin is formed at an optimum pH of 4.5-5.0. Below pH 3.6 no thiosulphinates are formed as alliinase is completely and irreversibly inhibited. As stomach acid has a low pH, a stomach acid resistant coating to protect alliinase would be required for ingested garlic powder preparations (Lawson and Hughes, 1992).

Although allicin is a relatively unstable compound, it is reasonably stable with a half-life of about 4 days at room temperature when stored in water. Allicin is less stable within garlic homogenate than in water. Stability of allicin within garlic homogenates increases with dilution. Allicin is an unstable compound mainly because it reacts easily with itself to form vinylthiophene, ajoene and about 40 unidentified compounds (Lawson, 1995). Thus dilution, especially in water, increases stability. Stability is thought to be further increased in water due to hydrogen bonding between water molecules and the oxygen atom present in allicin. Allicin is also most stable at low temperatures (Lawson, 1993). At room temperature, aqueous allicin is transformed to mainly diallyl trisulphide, diallyl disulphide and allyl methyl trisulphide (Iberl et al, 1990). However, within garlic homogenate the trisulphides are formed first followed by a slower transformation to more stable disulphides and tetrasulphides. In solvents such as methanol and chloroform, the half life of allicin is reduced to 48 hours, and to 24 hours in ethanol or acetonitrile (Lawson, 1993).

1.5.4 Antibacterial mode of action of allicin

It has been known from the early work of Cavallito and co-workers that allicin reacts rapidly with sulphhydryl compounds. Since allicin reacts readily with cysteine to produce S-allylmercaptocysteine an early hypothesis was that allicin inhibits

bacterial growth by reacting with essential bacterial –SH groups either by reacting with the thiol groups of enzymes or with cysteine residues on elongating polypeptide chains during protein synthesis (Cavallito, 1946). Confirmation that the thiosulphinate link (-SO-S) of allicin was essential for bactericidal action was provided by Small, Bailey and Cavallito (1947). Furthermore, addition of thiol compounds such as cysteine was shown to react with allicin and reverse bacteriostasis (Bailey and Cavallito, 1948). The explanation was that allicin reacts with cysteine allowing bacterial enzymes normally inhibited by allicin to remain active.

Enzymes containing sulphydryl (-SH) groups have been tested for sensitivity to allicin (Rao et al, 1946). Wills (1956) examined a variety of different enzymes and found that 11 out of 15 enzymes inhibited by allicin contained -SH groups whereas of the 15 enzymes tested that were resistant to allicin, only three were sulphydryl enzymes. Wills also confirmed that addition of cysteine protected bacterial cells from the inhibitory action of allicin.

Inhibitory activity of allicin against yeast has also been reported. Barone and Tansey (1977) hypothesised that even subinhibitory concentrations of allicin would affect cell division of *C. albicans* by inhibiting disulphydryl reductase, an NADPH – dependant flavoprotein, which reduces yeast cell wall disulphides to the thiols necessary for budding. Interactions between allicin and –SH groups may also induce the formation of mycelia. Ghannoum (1988), investigated that activity of an aqueous garlic extracts (AGE) against *C. albicans*. Scanning electron microscopy and cell leakage studies revealed that AGE affects the structure and integrity of the outer surface of yeast cells. Studies of the affect of AGE on yeast lipids showed that in the

presence of AGE the total lipid content of *C. albicans* was decreased with higher levels of phosphatidylserines and lower levels of phosphatidylcholines. Changes in fatty acid concentrations such as increased concentrations of palmitic and oleic acids, but lower concentrations of linoleic and linolenic acids, were also observed. There were also increases in the accumulation of esterified sterol glycosides. Ghannoum's study confirmed the earlier work of Adetumbi and co-workers who had shown that garlic blocks lipid synthesis of *C. albicans* (Adetumbi et al, 1986). Thus, the evidence suggests that AGE affects the total lipid and fatty acid content of *C. albicans*. Finally, allicin has also been shown to inhibit the growth of the protozoan parasite *Entamoeba histolytica* by inhibiting the activity of the trophozoites cysteine proteinase (Ankri et al, 1997).

It has also been proposed that the antimicrobial activity of allicin is not solely based on activity as a sulphydryl reagent. Changes in the DNA, RNA and protein synthesis of *Salmonella typhimurium* have been reported in the presence of allicin. Protein synthesis was reduced to 40% of the control value ten minutes after addition of allicin, but recovered to reach normal levels after 50 minutes. DNA synthesis was also only transiently and partially inhibited by allicin and restored to 100% after only 35 minutes contact. RNA synthesis was, however, much more profoundly inhibited by exposure to allicin. Only five minutes after allicin addition, RNA expression was merely 1% of the control value. However, expression began to recover after 40 minutes, and after 50 minutes reached 73% of the control value accompanied by bacterial growth. Three phases of inhibition of *S. typhimurium* by allicin were evident; a lag phase, an inhibition phase and then reduced resumption of growth. The lag period corresponds to the time between chromosome replication and cell

division, thus cells that have completed DNA synthesis before allicin addition are able to proceed through cell division. The inhibition of cells undergoing chromosome replication can be attributed to the almost total inhibition of RNA synthesis, as DNA synthesis requires RNA polymerase. *S. typhimurium* resumed growth 50 minutes after addition of allicin, however growth was at only 55% of the uninhibited control. Allicin was shown to be stable in the LB broth used in this experiment for more than four hours therefore, as these cells do not totally recover from allicin inhibition it can be speculated that allicin causes irreparable damage to the cell. Furthermore, as resumption of growth was most closely associated with resumption of RNA synthesis, it was assumed that this was the primary target of inhibition (Feldberg et al, 1988).

1.5.5 Antimicrobial spectrum of garlic

Garlic extracts have been tested for inhibitory activity against a wide array of micro-organisms. Studies have ranged from investigation of inhibition of single species to evidence of broad-spectrum anti-microbial activity against a range of Gram-positive and Gram-negative bacteria. Dehydrated garlic extracts have been shown to possess inhibitory activity against *S. typhimurium* and *Escherichia coli* (Johnson and Vaughn, 1969). Production of enterotoxins and growth of *S. aureus* is inhibited in the presence of dehydrated garlic (Gonzalez-Fandos et al, 1994). AGE has also been demonstrated to have bactericidal activity against *Helicobacter pylori* (Cellini et al, 1996) and *Mycobacterium avium* complex (Deshpande et al, 1993). Other studies, including those carried out by Cavallito and Bailey (1944), Rees and

co-workers (1993) and Didry and co-workers (1987) have emphasised the wide ranging antibacterial activity of garlic extracts. Garlic extracts have also been shown to inhibit many pathogenic species of fungi including *Aspergillus* (Pai and Platt, 1995) and *Candida* (Barone and Tansey, 1997; Ghannoum, 1988) species. In vitro studies have shown that intra-venous administration of commercial garlic preparations increase the anti-*Cryptococcus neoformans* activity of human plasma (Davis et al, 1990). Allicin preparations have also been shown to have considerable germicidal activity against protozoan species such as *Entamoeba histolytica*, the causative agent of amoebic dysentery (Ankri et al, 1997).

1.5.6 Metabolism and pharmacokinetics of constituents of garlic

Allicin is not found in the blood of individuals who have ingested garlic. No detectable serum or urine levels of allicin, or its metabolites ajoene, vinylthiins or diallyl sulphides were found in volunteers who ingested up to 25g of raw garlic (Lawson, 1993). Experiments have also been described in which allicin was added to blood plasma and then immediately extracted with chloroform. The results showed that allicin was not recovered unless the plasma was first deproteinated suggesting that allicin reacts with protein in the bloodstream. The half-life of allicin in fresh whole blood at 37°C is less than one minute (Lawson, 1993). It seems likely that allicin reacts in vivo with protein bound cysteine to produce the temporary metabolite S-allylmercaptocysteine. This compound then quickly reacts with blood cells to form allyl mercaptan. To account for the activity of garlic preparations as antimicrobial agents and the in vivo instability of allicin in the bloodstream, it has

been suggested that allyl mercaptan or a further metabolite of this compound is responsible for the therapeutic activity of garlic preparations (Shashikanth et al, 1985; Davis et al, 1990).

1.6 Oil extracts of plants

An essential oil is a mixture of organic compounds obtained from a single botanical source. Such oils can be extracted by either distillation or cold pressing. Use of distilled oils dates back only 1000 years to when the process of distillation was invented. However, the plants from which essential oils are derived have been used as medicines for much longer. Essential oils are not obtained readily from all plants. Typically, fresh plant material yields 1% to 2% of its weight as essential oil. The oils, however, can be obtained from a variety of plant materials including flowers, leaves, seeds, grasses, roots, rhizomes, woods, barks, gums, tree blossoms, bulbs and dried flower buds.

Oils from the same plant variety can contain different quantities of chemical compounds. This variation can be due to many factors including, the age of the plant, the time of year or even day upon which it is harvested. Environmental and other cultural conditions which affect plant growth, such as geographical location, elevation, weather conditions and soil type may also effect the quality of oil produced (Cruz et al, 1993). Differences in extraction techniques and the equipment used can also affect the composition of oil obtained. Oxidation and subsequent loss of activity of compounds present within essential oils can occur over time. This process occurs more rapidly in incorrectly stored oils. To preserve activity, oils should be stored away from heat and light (Tisserand and Balacs, 1995).

1.6.1 Antimicrobial activity of plant essential oils

Although spices and essential oils have been used since antiquity it was not until the 1880's that the first scientific studies of plant essential oils were published. In 1887, Chamberland reported the antimicrobial activity of oils in their gaseous and liquid states against anthrax bacteria. Cinnamon, marjoram, sandalwood, clove and juniper were found to be the most active of the oils tested. Cadeac and Meunier in 1889 published their findings, that cinnamon and clove were the most inhibitory of various essential oils tested against *Salmonella typhi* and *Burkholderia mallei*. The potential use of essential oils as food preservatives was recognised in 1911 by Hoffman and Evans, who observed that cinnamon, cloves and mustard prevented bacterial growth (Bartels, 1947). By 1925, the antimicrobial potential of individual compounds present within essential oils was recognised. Myers and Thienes, discovered that thymol, a compound present in thyme and oregano essential oils, could kill a yeast-like organism which caused dermatitis after only one minute of exposure; in contrast, 60 minutes exposure of 1% phenol was required to produce the same effect (Myers and Thienes, 1925). Myers, later showed that carvacrol, a compound also present in thyme essential oil, was capable of the same rapid killing of the same 'yeast-like organism'. Nine other yeast-like organisms were also studied. All were killed within one minute's exposure to thymol; however, cinnamon and clove oils required at least 25 minutes exposure (Myers, 1927). Kingery and Adkisson (1928) also reported the fungicidal activity of thymol and the oils of cinnamon and cloves. Recently, there has been renewed interest in the antimicrobial properties of essential oils and spice extracts. Due to the increasing prevalence of micro-organisms

exhibiting resistance to conventional antibiotics, several studies have been carried out to investigate the susceptibility of a number of bacteria, yeasts and fungi to essential oils. Some studies have demonstrated susceptibility against challenging pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci to tea tree, lavender, mint and thyme oils (Nelson, 1997). More extensive studies have involved screening of over fifty plant essential oils for antibacterial activity against twenty five genera of bacteria (Deans and Ritchie, 1987) or against various bacterial, fungal and yeast species (Hammer et al, 1999; Farag et al, 1989; Yousef and Tawil, 1980). Oils of bay, cinnamon, clove, oregano and thyme were the most consistently active of the oils tested in these studies. Numerous other studies have concentrated on the potential use of essential oils as food additives to prevent bacterial spoilage. This strategy is by no means novel. Mustard which the Romans used to preserve grape juice from the growth of fermenting organisms is believed to have derived its name from must, newly fermented grape juice. Renewed interest in the addition of plant extracts to foodstuffs has been generated since synthetic food additives are no longer perceived as safe by the consumer (Shelef, 1983). Several investigators have reported the antimicrobial activity of essential oils against foodborne pathogens such as *Campylobacter jejuni*, *Salmonella enteritidis*, *Listeria monocytogenes*, *S. aureus*, *E. coli* (Smith-Palmer et al, 1998; Aureli et al, 1992; Pasteur et al, 1990; Farbood, 1976) and *Vibrio parahaemolyticus* (Beauchat, 1976). The most inhibitory oils were revealed as those from oregano, thyme, cinnamon, clove, rosemary and garlic. Gram-positive bacteria appeared more susceptible than Gram-negative bacteria to the oils (Smith-Palmer, 1998; Shelef, 1983; Farbood, 1976). The susceptibility of food spoilage

yeasts to the antimicrobial activity of essential oils has also been noted (Conner and Beauchat, 1984). For practical purposes it is important to note that higher concentrations of essential oil are required in foods than in laboratory media to inhibit growth of micro-organisms (Farbood, 1976).

Investigators have also highlighted the potential use of essential oils in cosmetics and soaps (Nadal et al, 1973; Maruzzella and Henry, 1958). However, when oils active against *S. aureus*, *E. coli* and *C. albicans* in laboratory media were added to soap, antimicrobial activity was not observed (Morris et al, 1979). The activity of antimicrobial compounds present within the oils maybe inhibited by soap, thus rendering them ineffective in these conditions.

1.6.2 Use of essential oils in dentistry

Essential oils, particularly oils of cloves and thyme have been used in dentistry since the late 19th century. Oil of cloves contains high concentrations of the phenol, eugenol whilst thyme oil comprises mainly the phenols thymol and carvacrol. Thymol and carvacrol can also be extracted from caraway, marjoram, oregano, rosemary and savory plants. The first reported use of eugenol in dentistry was in 1837, when Bonastre used a mixture of eugenol and magnesium oxide as a filling material. Dentists have also used thymol and carvacrol since the 19th century. In 1885, Gorgas reported the use of thymol and carvacrol as a treatment for suppuring pulps, alveolar abscesses, stomatitis and odontalgia. The use of thymol for cavity sterilisation was discontinued in the first half of the 20th century, however its use is still evident in dentistry today as one of the main ingredients of Listerine® (Meeker,

1988). A more recent, scientific examination of the effectiveness of various essential oils and their component compounds against some of the important pathogenic micro-organisms found in the mouth including, *Streptococcus mutans*, *S. aureus*, *Klebsiella pneumoniae*, and *Actinomyces viscosus*, found thyme oil to be the most active. The most active essential oil ingredients were found to be thymol followed by carvacrol and eugenol (Meeker, 1988). In a separate study, fifteen oral bacteria were challenged with essential oils and oil components. Thymol was the only substance to be active against all the bacteria surveyed and once again was found to be more active than eugenol (Shapiro et al, 1994).

1.6.3 Essential oil composition

Each essential oil typically comprises a number of organic compounds many of which may be present in the essential oils of more than one plant. Thyme oil can be extracted from *Thymus zygis* or *Thymus vulgaris*. Different chemotypes of thyme oil are obtained from this genus. Each chemotype of thyme oil has a different chemical composition, the most common of which is the thymol chemotype, composed of between 32% and 63% thymol and 1%-5% carvacrol. Others chemotypes include the carvacrol and wild thyme chemotypes which both contain more carvacrol than thymol, and the thymol / carvacrol chemotype made up of equal amounts of these compounds (Tisserand and Balacs, 1995).

Other essential oils tend to vary less dramatically in composition; in as much as one compound consistently dominates the oil. Bay oil, most commonly produced from *Pimenta racemosa* contains 38% to 75% eugenol. Cinnamon leaf oil from *Cinnamomum verum* also contains eugenol at a concentration of between 70 % and

90%. Clove oil from *Syzygium aromaticum* contains large amounts of eugenol and some iso-eugenol at concentrations of 70%–95% and 0.14%–0.23% respectively. Sweet fennel oil from *Foeniculum vulgare* contains mainly trans- anethole (75%-92%), but also contains small quantities of estragole and cis-anethole. *Melaleuca alternifolia* produces tea tree essential oil which contains between 27%-58% terpinen-4-ol, a terpene alcohol. Other major components of tea tree oil are the monoterpenes α -terpinene and γ -terpinene. Garlic produces mainly diallyl disulphide along with a large number of other sulphur containing compounds.

Phenols

Most essential oils contain many different compounds but it is the one or two major components that largely shape the pharmacology of the oil. Many of the most commonly used oils are composed of phenols such as eugenol and the isomers thymol and carvacrol. The chemical structure of phenol contains a benzene ring with a highly reactive -OH group attached directly to the ring. Other chemical groupings, commonly found in essential oils include ethers, such as anethole and estragole, or terpenes and terpene alcohols that are built on the isoprene unit commonly found in plant biochemistry.

Phenol was first described by Runge in 1834, but it was not until 1860 that Kuchenmeister advocated the use of phenol as an antimicrobial agent (Bennet, 1959). In 1875, Lister introduced a phenol spray as the first general disinfectant in medical history (Meeker, 1988). Phenols are more active at elevated temperatures and acid pH. They partitioned from aqueous phase to lipoidal phase; thus, soaps can interfere with the activity of phenols by creating an additional lipoidal phase, reducing the

concentration available to inhibit bacteria. The presence of blood, serum, alcohol and salivary mucus all reduce the activity of phenols. Dead bacterial cells absorb phenol and thus reduce the amount available to inhibit live cells (Bennet, 1959). Phenols and other cyclic hydrocarbons exert their bactericidal effect by disrupting the bacterial cell membrane (Judis, 1963). Hydrophobic reactions of phenols with the cell membrane affect membrane embedded proteins and membrane function. In the presence of cells, these lipophilic compounds partition into the membrane where their accumulation leads to swelling of the lipid bilayer and subsequent increased membrane permeability (Sikkema et al, 1994). Ultimately cell contents are lost through the damaged cell membrane (Judis, 1963). The biological activity of membrane proteins may be altered by hydrogen bonding of the highly reactive OH group of phenols with the active site of membrane bound enzymes (Farag, 1989; Sikkema, 1994). As thymol, carvacrol and eugenol are all phenols, the major antimicrobial mechanism by which essential oils containing these compounds act is most likely to be by destruction of the bacterial cell membrane. Finally, it is important to consider that a number of chemical constituents, each of which may exert antimicrobial activity by different mechanisms, acting against different targets within the bacterial cell, may work together within essential oils in 'natural synergy' to kill bacterial cells.

1.7 Endogenous antimicrobial peptides

A variety of antimicrobial substances which act as endogenous antibiotics are produced by humans, animals, insects and plants. Thus, it would appear that antimicrobial peptides of less than 100 amino acids form an intrinsic part of innate

host defences. Most of these peptides are cationic, *i.e.* they have a net positive charge and amphipathic, *i.e.* they are composed of hydrophilic and hydrophobic regions. For the purpose of this thesis, discussion will be limited to the major groups of antimicrobial peptides expressed in humans.

1.7.1 Defensins

Defensins are a family of antimicrobial peptides which have been identified in plants, animal and humans. As well as their antimicrobial properties, some of these peptides also have a role in inflammation. Defensins share structural similarities. They contain six cysteine residues which form three intramolecular disulphide bridges. Human defensins are divided into α -defensins and β -defensins based on how the cysteine residues are paired into disulphide bridges. α -defensins are linked 1-6, 2-4 and 3-5 (Hill et al, 1991) whereas β -defensins are linked 1-5, 2-4 and 3-6 (Zimmerman et al, 1995). Human α and β -defensins are encoded by a cluster of genes on chromosome 8p23 and are thought to have a common genetic ancestry (Sparkes et al, 1989; Bevins et al, 1996; Liu et al, 1997; Harder et al, 1997).

α -defensins

α -defensins were first described in 1985 (Ganz et al, 1985) and six have been identified to date in humans. Four such peptides are found within the azurophilic granules of the neutrophil (HNP 1-4) (Lehrer et al, 1993). The two other α -defensins identified to date, HD-5 and HD-6, are found in the secretory granules of

the intestinal Paneth cells (Jones and Bevins, 1992; 1993). HD-5 is also found in the epithelial cells of the female reproductive tract (Quale et al, 1998).

Defensins HNP 1, 2 and 3 make up 30-50% of the protein present in azurophilic granules, constitutes 5-7% of total neutrophil protein. HNP-4, however, constitutes only 1% of the total defensin content. When phagosomes containing engulfed microbes fuse with these granules, the defensins bind to the membrane and associate into multimers to form channels, which disrupt the integrity of the microbial cell membrane, and through which more defensin can pass into the cell (Lehrer et al, 1993).

α -defensins can also perform other activities as part of the immune response. For example, defensins have been shown to stimulate the release of cytokines from airway epithelial cells (Van Wetering et al, 1997) and T cells (Lillard et al, 1999) and to play a role in the attraction of monocytes, (Territo et al, 1989) macrophages, granulocytes and lymphocytes (Welling et al, 1998) into inflammatory sites.

β -defensins

The first β -defensin to be identified was tracheal antimicrobial peptide (TAP) isolated from bovine trachea (Diamond et al, 1991). Shortly after the discovery of TAP, Selsted and co-workers identified 13 other antimicrobial peptides from bovine neutrophils that shared high sequence similarity with TAP, but differed from α -defensins (Selsted et al, 1993). These peptides were named β -defensins. It was not until 1995 that the first human β -defensin was identified in human plasma (Bensch et al, 1995). Subsequently, this defensin was also shown to be present in the epithelial cells of various human organs including the lung (M^cCray and Bentley,

1997; Goldman et al, 1997) and urinary and female reproductive tracts (Valore et al, 1998). The second human β -defensin, hBD-2, was identified two years later from psoriatic scales of human skin. Expression of this defensin was also identified in the lung (Harder et al, 1997; Bals et al, 1998).

The activity of hBD-1 and hBD-2 was reported to be salt sensitive (Goldman et al, 1997; Bals et al, 1998). However, hBD-1 isolated from human urine was found to maintain its antimicrobial activity against *E. coli* in both dilute and normal urine suggesting that not all forms of hBD-1 are salt sensitive (Valore et al, 1998). ASF isolated from primary cultures of airway epithelial cells of non-CF individuals has been shown to have antimicrobial activity which was absent in similar secretions from CF airway epithelial cells (Smith et al, 1996). Investigators had previously reported that concentrations of Na^+ and Cl^- were raised in CF compared to non-CF airways (Joris et al, 1993). This led to the possibility that inactivation of β -defensins by the high salt levels in CF ASF might in some part explain the susceptibility of CF patients to chronic bacterial infections. As β -defensins are structurally similar to α -defensins, it is likely that β -defensins also kill microorganisms by disrupting the cell membrane. Homologues of hBD-1 and hBD-2 which have salt sensitive activity against *E. coli*, *S. aureus* and *P. aeruginosa* have been described in mice (Morrison et al, 1998; Bals et al, 1998b; Morrison et al, 1999). Interestingly, the inhibitory activity of hBD-1 against *P. aeruginosa* is considerably greater than the activity of the murine homologue mBD-1.

1.7.2 Cathelicidins

Cathelin is an acronym for cathepsin L inhibitor and peptide antibiotics which when synthesised are preceded by a cathelin-containing domain, are called cathelicidins. To date, humans are known to express only one cathelicidin, LL-37. The cathelin containing prepropeptide hCAP-18 is cleaved by neutrophil elastase to release the α -helical antimicrobial peptide LL-37. Expression of this peptide has been identified in the human lung (Bals et al 1998c). Synthetic LL-37 is active against Gram-positive and Gram-negative organisms and exhibits some salt sensitivity at NaCl concentrations of 100mM (Turner et al, 1998). When LL-37 activity against *E. coli* was examined by Bals and co-workers, salt sensitivity was noted through the 20 and 300mM range of NaCl examined (Bals et al, 1998c).

1.7.3 Granulysin

Granulysin, a protein present in the granules of human T cells and natural killer cells is a member of the saposin-like protein family. Recombinant human granulysin, has been shown to have inhibitory activity against a broad spectrum of micro-organisms (Stenger et al, 1998).

1.7.4 Histatins

Histatins are histidine rich proteins, present in human saliva, which have some activity against *C. albicans* (Tsai et al, 1998). The mechanism of action of these peptides appears to differ from that of other antimicrobial peptides as loss of membrane integrity was shown to occur after cell death and not as a result of histatins disrupting the yeast cell membrane. Activity of histatins may be linked to the binding of a 67kDA protein of *C. albicans* (Edgerton et al, 1998).

1.7.5 Antileukoproteases (ALP)

The antimicrobial activity of two proteinase inhibitors of the ALP family has recently been described. Both secretory leucocyte proteinase inhibitor (SLPI) and elafin (also known as elastase specific inhibitor or skin derived antileukoprotease) were originally described as inhibitors of serine proteases secreted by neutrophils such as human neutrophil elastase (HNE) and cathepsin G. Serine protease inhibitors neutralise proteolytic enzymes such as HNE when the protease is released from neutrophils during phagocytic destruction of micro-organisms, thus preventing damage to the host. Both inhibitors are produced locally in human airways (Sallenave et al, 1999).

SLPI

SLPI is a 107 amino-acid long protein that contains sixteen cysteines which form eight disulphide bridges (Seemuller et al, 1986; Grutter et al, 1988). SLPI has two domains. The COOH-terminal domain contains the antiproteinase site (Kramps et al,

1990; Micklein et al, 1990; Eisenberg et al, 1990) and the NH₂-terminal domain is responsible for the antimicrobial activity and for stabilising complexes between SLPI and elastase (Hiemstra et al, 1996; Ying et al, 1994). Whole recombinant ALP and the isolated NH-terminal domain have been reported to have salt-sensitive antibacterial activity against strains of *E. coli* or *S. aureus* (Hiemstra et al, 1996).

Elafin

Elafin is 95 amino acids long and is also composed of two domains, the COOH-terminal proteinase domain and the NH₂-terminal domain. Elafin has less than 40% sequence homology with the COOH-terminal domain of SLPI but, like SLPI, contains eight cysteines arranged into four disulphide bonds (Wiedow et al, 1990; Tsunemi et al, 1992). Elafin has greater specificity in proteinase inhibition than SLPI, inhibiting only human neutrophil elastase, porcine pancreatic elastase and proteinase-3 (Sallenave and Ryle, 1991; Wiedow et al, 1991). Recently, synthetic elafin has been reported to have antibacterial activity against *P. aeruginosa* and *S. aureus* (Simpson et al, 1999).

The aim of this project was to investigate novel antimicrobial strategies against *B. cepacia*. The project focused on two major themes. First, a study of antimicrobial compounds present in plants, in particular compounds from garlic (*Allium sativum*) and thyme (*Thymus vulgaris*). Second, antimicrobial activity of cationic peptides present in human airway secretions such as human β -defensin-1 and elafin was examined.

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial isolates

Twenty isolates of *Burkholderia cepacia* from the collection of Prof. John Govan and listed in Table 2.1, were used throughout this thesis. All of these isolates were from different patients or environments except strains C1963 and C1964, which were isolated from the sputum of the same patient. Additional bacterial strains also used are listed in Table 2.2.

2.1.2 Media

Unless otherwise stated media were provided by Oxoid Ltd., Basingstoke, Hampshire, England. All media were prepared in distilled water and sterilised by autoclaving at 121°C at 15 psi for 15 minutes.

Nutrient agar: Columbia agar base, 39g/L

Cepacia isolation agar (CIA): 32.5g/L (MAST Diagnostics, Bootle, Merseyside) plus one selectab (MAST) per 100ml of agar added after autoclaving and once the agar has cooled to 50°C. This gives a final antibiotic concentration of 100µg/ml ticarcillin and 300 units/ml of polymyxin B.

Pseudomonas isolation agar (PIA): 45g/L (Difco Laboratories, Detroit, Michigan, U.S.A.) plus 2% (v/v) glycerol (Sigma) added prior to autoclaving.

Blood agar: Columbia agar base plus 5% defibrinated horse blood added after autoclaving and once agar has cooled to 50°C.

Iso-sensitest agar (ISA): 31.4g/L.

Nutrient broth (NYB): Nutrient broth no.2 plus 0.5% yeast extract (Difco)

Iso-sensitest broth (ISB): 23.4g/L

Skimmed milk: Skimmed milk powder 10% (w/v) sterilised for 5 minutes.

Saline: 0.85% sodium chloride (w/v) (Sigma-Aldrich Coy. Ltd., Poole, Dorset, UK)

Phosphate buffered saline (PBS): One PBS tablet per 100ml distilled water.

Table 2.1. Isolates of *B. cepacia* used in this thesis.

<u>Isolate</u>	<u>Description</u>	<u>Genomovar</u>
Isolated from CF patients		
C1052	Sputum, Edinburgh, non epidemic	II
C1511	Sputum, Manchester epidemic strain	III
C1576	Sputum, Glasgow epidemic strain	II
C1632	Sputum, Newcastle epidemic strain	III
C1963	Sputum, Edinburgh, non epidemic, non mucoid	I
C1964	Sputum, Edinburgh, non epidemic, mucoid	I
J415	Sputum, Edinburgh, non epidemic	III
J419	Sputum, Edinburgh, non epidemic	I-III
J2315	Sputum, Edinburgh epidemic index case, ET12 lineage	III
A507	Sputum, Edinburgh, non epidemic	III
Non-CF clinical isolates		
C1773	Blood culture, Papworth	III
C1962	Cerebral abscess, Aberdeen	II
J2503	Wound infection, ATCC 25608	III
J2524	Urinary tract infection, ATCC 17765	III
Environmental isolates		
J673	Onion, type strain, ATCC 25416	I
J2502	ATCC 10856	I-III-IV
J2421	Soil, Canada, ATCC17616	II
J2537	Soil, RBGE	I-III-IV
J2552	Soil, RBGE	I
J2742	Biological control isolate from pea rhizosphere	V

Table 2.2. Other bacterial isolates used in this thesis.

Strain	Description
<i>Staphylococcus aureus</i>	
C1705	Clinical CF strain
J2407	ATCC 29213
<i>Escherichia coli</i>	
J2408	ATCC 25922
<i>Pseudomonas aeruginosa</i>	
J1385	Non muciod CF strain
PAO1	Non CF clinical strain, classic lab strain
NCTC 10662	Susceptibility testing control strain
<i>Burkholderia cepacia</i>	
C3055	K61-3, <i>opcM</i> PCR control

All chemicals were provided by Sigma-Aldrich Coy. Ltd., Poole, Dorset, UK unless otherwise stated.

2.1.3 Buffers for pulsed field gel electrophoresis

SE Buffer: 4.38g NaCl plus 9.3g EDTA Na₂ dissolved in 900ml distilled water.

The pH was adjusted to 7.5 and the solution made up to 1 litre. The buffer was then filter sterilised and stored at 4°C.

Lysis Buffer: 1g N-lauroylsarcosine plus 18.6g EDTA Na₂ was added to 80ml of distilled water. 10ml NaOH was added to dissolve the EDTA Na₂. The pH was adjusted to 9.5 and the solution made up to 1 litre. The buffer was then filter sterilised and stored at room temperature.

TE Buffer: 1.21g Tris (hydroxymethyl)-aminomethane (Bio-Rad, Hercules, California, U.S.A.) plus 3.72g EDTA Na₂ was dissolved in 900ml of distilled water. The pH was adjusted to 7.5 and the solution made up to one litre. The buffer was then filter sterilised and stored at 4°C.

2.2 METHODS

2.2.1 Storage and recovery of isolates

Bacteria were stored at -70°C in 1ml of 10% w/v skim milk. After thawing, *B. cepacia* were inoculated onto nutrient agar, incubated overnight at 30°C and subcultured on CIA at approximately four day intervals. Strains of *P. aeruginosa* were inoculated onto nutrient agar and subcultured on PIA at approximately weekly intervals. The *S. aureus* and *E. coli* strains were recovered and subcultured weekly on nutrient agar.

2.2.1 Pulsed Field Gel Electrophoresis

Preparation of unsheared DNA in agarose plugs

Isolates of *B. cepacia* were inoculated into 10ml volumes of nutrient broth and incubated overnight at 37°C in an orbital incubator. These cultures were then centrifuged at 4000 x g for 10 minutes, the supernate discarded and the remaining bacterial pellet re-suspended in SE buffer until standardised to an optical density of 1.5 at 590nm by spectrophotometry (Camspec, Sawston, Cambridge, England.). 1% low melting point agarose (Bio-Rad) was prepared in SE buffer. 500µl of this agarose was then added to 500µl of bacterial suspension in a sterile microcentrifuge tube. The mixtures were then dispensed into the slots of a plug mould to create one plug for each of the bacterial isolates. Once the agarose had set the plugs were transferred to sterile bijou bottles.

Lysis of bacteria within plugs

2ml of lysis buffer containing 0.5% triton-X 100 was dispensed into each bijou. The plugs were then incubated overnight at 56°C in a waterbath. The lysis buffer was then removed and replaced with 2ml of TE buffer and the plugs left for 30 minutes at 4°C. This step was repeated twice before the plugs were left overnight at 4°C.

Restriction of bacterial DNA within plugs

A 2.5mm by 9mm portion of each plug was cut and placed in a sterile microcentrifuge tube. 90µl of sterile distilled water and 10µl of 10x reaction buffer 2 (Gibco BRL, Life Technologies Ltd., Paisley, Renfrewshire, Scotland) was then

added to the tubes and the plugs left at 4°C for 30 minutes. The buffer was then removed and replaced with 90µl of sterile distilled water, 10µl of 10x reaction buffer 2, 2µl of 10mg/ml bovine serum albumin (Promega, Madison WI, U.S.A.), 2µl dithiothreitol and 2µl of the restriction enzyme Xba1 (Gibco BRL). The contents of the microcentrifuge tubes were mixed gently and incubated in a 37°C overnight. The next day the enzyme mixture was removed and replaced with TE buffer, while 100ml of pulsed field gel agarose (Bio-Rad) was prepared in 0.5 x TBE buffer (Gibco BRL). Once the agar had cooled to 60°C, most was poured into a gel mould and allowed to set. The plugs were then loaded into the gel and sealed in with the remaining agarose. The gel was then placed into the tank of the CHEF – DR II electrophoresis system (Bio-Rad) and covered with 2 litres of TBE buffer. The gel was then run with an initial pulse time of 2.9 seconds and a final pulse time of 35.4 seconds for 20 hours at 200 volts and at a temperature of 14°C. Following electrophoresis, the gel was stained in 1µg/ml ethidium bromide (Bio-Rad) for 15 minutes and destained twice in distilled water to enable the DNA bands to be viewed within the gel by UV transillumination and recorded photographically.

2.2.3 Antibiotic susceptibility testing

Broth macrodilution method

Twenty isolates of *B. cepacia* and the control strain of *P. aeruginosa* (NCTC 10662) were inoculated into 10ml volumes of ISB and incubated for 18 hours at 37°C with shaking. After this time, each culture contained approximately 1×10^9 cfu/ml, as verified by viable counts performed on five selected strains. The cultures were then diluted in ISB to a concentration of approximately 1×10^7 cfu/ml.

Stock solutions of the antibiotic to be tested were made up at concentrations of 1mg/ml, 100µg/ml, 10µg/ml and 1µg/ml. These stock solutions were then added to 10ml aliquots of ISB to give final concentrations of doubling dilutions between 32µg/ml and 0.03µg/ml.

100µl of bacterial culture was then added to the broth aliquots containing antibiotics, to give a final concentration of approximately 1×10^5 cfu/ml.

Controls containing no antibiotics were also prepared for each strain.

The tubes were incubated for between 16 and 18 hours at 37°C with shaking before the MIC was determined. The MIC was taken to be the lowest concentration of antibiotic at which there was no visible growth. Individual experiments were considered valid if the MIC for the control strain of *P. aeruginosa* was within one twofold dilution of the expected result as set out in Phillips et al (1991).

Agar dilution method

Stock solutions of antibiotics and cultures to be tested were prepared as in the 'broth macrodilution method' previously described. Plates were prepared containing antibiotics at concentrations of between 32µg/ml and 0.03µg/ml in doubling dilution steps. The correct volume of antibiotic was added to 20 ml of ISA (cooled to 60°C) in a Petri dish. A control plate containing no antibiotics was also prepared. The plates were swirled gently to mix and the agar left to set.

Once the plates had been dried they were inoculated with 1µl of a diluted broth culture of each isolate delivered by a multipoint inoculator (Denly, Billingshurst, Sussex) and providing a final inoculum of approximately 10^4 cfu. The plates were then incubated for 16-18 hours at 37°C before the MIC was determined. The MIC

was once more defined as the lowest concentration at which there is no visible growth; in this assay system one or two colonies or hazes of growth were disregarded.

2.2.4 Determination of minimum inhibitory concentration and minimum bactericidal concentration of plant extracts

Broth cultures of 20 *B. cepacia* isolates and control strains of *S. aureus* (J2407), *E. coli* (J2408) and *P. aeruginosa* (NCTC 10662) were prepared at a concentration of approximately 1×10^7 cfu/ml as set out in the broth macrodilution method. Plant extracts were added to 10ml aliquots of ISB to give final concentrations of between 6% and 0.05% for aqueous garlic extract and between 0.25% and 0.01% for the plant oils. 100µl of bacterial culture was then added to the broth aliquots to give a final bacterial concentration of approximately 1×10^5 cfu/ml. Controls containing no plant extract were also prepared for each bacterial strain. The contents of each tube were mixed for 30 seconds to ensure the plant extract had been evenly dispersed through the broth. The tubes were then incubated at 37°C with shaking for 16-18 hours. 100µl was then sampled from each tube and spread over a plate of nutrient agar. Tenfold dilutions were also made of the tube containing the lowest concentration of plant extract that did not appear turbid. One hundred fold dilutions were made of the control tube. 100µl of each of these dilutions was spread over plates of nutrient agar and were incubated for 48 hours at 37°C to obtain a count of the number of viable organisms remaining. Due to the limitations of the serial dilution technique it was not possible to detect less than 10 cfu/ml.

2.2.5 Synthesis of aqueous garlic extract

AGE was prepared by a modification of the extraction procedure outlined by Pai and Platt (1994). A known weight of peeled, chopped garlic cloves was added to a equal volume of distilled water in a Waring blender (Waring, New Hartford, Connecticut, U.S.A.) and blended for approximately 3 minutes at high speed. The homogenised garlic was then strained through two layers of muslin to remove any large debris. The liquid obtained was then centrifuged at 20,000g for 40 minutes at 4°C in a Sorvall centrifuge.

The supernatant was then sterilised by Seitz filtration under a pressure of 10 psi. Pai and Platt recommended storage for no more than one week at -70°C before use, however the extract was found to be active for up to one month when stored at -70°C.

2.2.6 Synergy testing by the checkerboard technique

The 'checkerboard' technique described by Garrod and Waterworth (1962) is commonly used to test synergy between conventional antibiotics. The technique was modified to test for synergy between AGE and four antibiotics commonly used to treat microbial lung infections of CF patients, ceftazidime, ciprofloxacin, chloramphenicol and meropenem. As the MICs for both AGE and the selected antibiotics had previously been determined, checkerboards were set up as shown in Figure 2.1.

Each square on the diagram represents a WR tube, in which the final volume equalled 1ml. Each tube contained 0.5ml of approximately 2×10^4 cfu/ml. of *B. cepacia*, 0.25ml of one concentration of AGE and 0.25ml of one antibiotic concentration. Each of the antimicrobials to be tested was initially prepared at a concentration 8 x the MIC, to achieve a concentration of 2 x MIC in 1.0ml, when only 0.25ml was added. The antibiotic suspensions were then diluted to give final concentrations of 1 x MIC, 0.5 x MIC, 0.25 x MIC and 0.12 x MIC and the checkerboard arranged accordingly. All dilutions of antibiotics and bacterial cultures were made in ISB to support the growth of the organisms. The ‘checkerboards’ were incubated at 37°C overnight.

Fig. 2.1 Diagram of a typical checkerboard.

Antibiotic concentration (A)	2.0 x MIC						
	1.0 x MIC						
	0.5 x MIC						
	0.25xMIC						
	0.12xMIC						
	0.0 x MIC						
		0.0	0.12	0.25	0.5	1.0	2.0
Garlic Concentration x MIC (G)							

The checkerboards were used to determine the amounts of garlic and antibiotic that alone or in various combinations produce a minimum inhibitory combination of antimicrobials. To visualise this, the amounts of garlic and antibiotic were plotted on

an arithmetic scale, as proportions of the MIC that, alone or in combination inhibit the growth of a particular strain of *B. cepacia*. If the line joining these points, known as an isobol, is straight this indicated an additive effect. If the isobol bowed upwards this indicated an antagonistic effect of the drugs in combination whereas if the isobol bowed downwards, synergy was indicated.

2.2.7 Killing curves

Preparation of static cultures

10ml aliquots of iso-sensitest broth were inoculated with the strain under investigation and incubated for 16-18 hours at 37°C with shaking. At this time, the number of organisms present in the culture was approximately 1×10^9 cfu/ml. This overnight culture was diluted in ISB until it contained approximately 1×10^7 cfu/ml.

Preparation of log phase cultures

10ml aliquots of iso-sensitest broth were inoculated with the strain under investigation. These suspensions were then incubated for 16-18 hours at 37°C with shaking and the bacteria then centrifuged at 4000 x g for 10 minutes and the pellet washed in 10ml of fresh prewarmed ISB. 100µl of this suspension was then used to inoculate a fresh prewarmed 10ml aliquot of ISB, and the culture incubated at 37°C with shaking until the bacteria reached the log phase of growth. Growth curves were produced for *B. cepacia* strains J2315 and J2421 to establish at what time after inoculation log phase growth occurs.

100µl of a 1×10^7 cfu/ml suspension of either static or log phase bacteria was added to 10ml of ISB to which the minimum bactericidal concentration of the plant extract to

be analysed had already been added. A control containing no plant extract was also prepared. The tubes were then incubated at 37°C with shaking. Killing curves with AGE were achieved by sampling at 0 hours and then hourly between eight and 20 hours, for both test and control tubes. Killing curves with thyme oil and thymol involved sampling at zero hours, 10 minutes, 20 minutes, 30 minutes, 45 minutes and one hour. Sampling was carried out in triplicate. At each time point 100µl of undiluted sample was spread directly onto a nutrient agar plate. Tenfold dilutions ($10^1, 10^2, 10^3$) were made and 100µl of each dilution was spread onto nutrient agar and the plates incubated for 48 hours 37°C to determine the number of surviving organisms.

2.2.8 Dose Response Curves

Cultures of the bacterial strains to be analysed were grown to log phase as set out above. 200µl of this bacterial suspension was added to 5mls of double strength broth to which 4.8mls of antibiotic solution, in sterile distilled water had been added. Antibiotics were added to give final concentration in 10mls of 0, 0.001, 0.01, 0.1, 1, 10 and 100 where 1 equals 1mg/ml for thymol, 1ml/ml for AGE and 1µl/ml for thyme oil. An initial count of viable bacteria was obtained from an average of three counts from the tube containing no antibiotics at time zero. The tubes were then incubated at 37°C for the same time the bacteria were initially incubated to reach their log phase of growth. Viable counts were then made from each tube and the number of surviving bacteria calculated as a percentage of the initial count.

2.2.9 DNA extraction

An aliquot of 500µl of an overnight broth culture was placed in a sterile microcentrifuge tube and spun in a microcentrifuge (Sanyo, Itasca, IL, USA) for 2 minutes at 15,000g. The supernatant was removed and the remaining pellet washed in 1 ml of 50mM Tris HCl (Bio-Rad), 20mM EDTA. The pellet was then resuspended in 500µl of 50mM of Tris HCL, 2mM EDTA with 100µg/ml of lysosyme and incubated for 15 minutes at 4°C. Five mg/ml SDS (BDH, Poole, Dorset, England) and 100µg/ml proteinase K was added to the suspension before incubation for a further 2 hours at 37°C.

The bacterial lysate was sequentially extracted with equal volumes of Tris saturated phenol, phenol:chloroform (1:1) and chloroform. In each extraction 0.5ml of solvent was added, the suspension mixed thoroughly and then spun down at 15,000g for 5 minutes. The aqueous layer was then removed into a fresh tube to which the next solvent was added.

50µl of 3M sodium acetate and two volumes of 95% ethanol (Mackay and Lynn, Edinburgh, Scotland) were then added and the suspension held overnight at -20°C. The suspension was centrifuged at 15,000g for 30 minutes, and the pellet washed in 70% ethanol. The pellets were then dried at room temperature to insure all the remaining ethanol was removed and resuspended in 200µl of 10mM Tris, 1mM EDTA.

To measure the purity of DNA present in the sample, optical densities at 260nm and 280 nm were compared. The ratio between the readings provides an estimate of the

purity of the nucleic acid; a pure preparation of DNA has a value of between 1.8 and 2.0. All the DNA preparations used for PCR had ratios of approximately 1.8.

2.2.10 Amplification of DNA by the Polymerase Chain Reaction (PCR)

DNA was extracted from 21 isolates of *B. cepacia* and from a control strain of *P. aeruginosa* (NCTC 10662), as described previously. Primers were selected to detect and amplify a 493bp piece of the *pen A* gene or a 564bp piece of the *opcM* gene of *B. cepacia*. The sequence of the primers used is shown below. A reaction mix was prepared for each isolate containing 34.5µl sterile distilled water, 5µl PCR buffer (Promega), 2µl of dNTP (Promega), 2µl of each of the primers (Cruachem, Glasgow, Scotland), 2µl of dimethyl sulfoxide (DMSO), 0.5µl of Taq polymerase (Promega) and 2µl of the DNA to be analysed. The reaction mix was then cycled automatically in a thermal cycler (Techne, Cambridge, UK) Thirty five cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C were performed. Each step lasted one minute. The PCR products were then removed from the thermal cycler and stored at 4°C.

The PCR products were analysed on a 1.5% agarose gel (Bio-Rad) run in 0.5% TBE buffer. 2µl of gel loading buffer, containing 0.05% bromophenol blue, 0.05% xylene cyanol and 25% glycerol was added to 10µl of PCR product and this mixture loaded into the well of the agarose gel. A 100 base pair marker (New England Biolabs, Beverly, MA, USA) was used to estimate the size of the PCR products. 1µl of marker was added to 11µl of sterile distilled water and 2µl of gel loading buffer and these markers run along side the PCR products on the gel. The gel was run at 50

volts for approximately 4 hours, then stained in 1µg/ml ethidium bromide (Bio-Rad) for 15 minutes and recorded photographically.

Pen A primers

Forward primer ¹⁵⁰³ATCTCGTCAACTATTCGCCC¹⁵²²

Reverse primer ¹⁹⁹⁵TGCGTGTAGTAGACGGCAAG¹⁹⁷⁶

OpcM primers

Forward primer ⁷⁰¹TACACGAAGGCGACATCAG⁷¹⁹

Reverse primer ¹²⁶⁴GATCATCGAGCAAACGCAG¹²⁴⁶

2.2.11 Analysis of plant extracts by High Performance Liquid Chromotography

Aqueous garlic extract, allicin, thyme oil and its major component thymol were analysed by reverse-phase high performance liquid chromatography (RP-HPLC) with Gilson 712 series chromatographic equipment (Gilson Plc, Middleton, WI, USA). Detection was at 240nm for AGE and allicin, and 275nm for thyme oil and thymol using a column with C₁₈ as the active group (Beckman, Fullerton, CA, USA; 5µm pore size Ultrasphere ODS; internal dimensions - 150 by 10 mm). Separation was performed using a gradient of acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid and HPLC grade water. Thyme oil and thymol were separated using a continuous linear gradient (0 to 100%) of acetonitrile. AGE was analysed using a different gradient of acetonitrile and water designed to give a clear separation of the components of this extract. The flow rate in both cases was 1.3 ml min⁻¹. Plant

extracts were filtered through a 0.2µm filter before injection to remove any particles that might damage the column. 100µl of plant extract was then added to 900µl of a 12% solution of acetonitrile and this 1ml sample injected into the column. The fractions corresponding to peaks shown on the detector were collected and the acetonitrile removed by evaporation.

These compounds were then tested for antimicrobial activity against *B. cepacia* strain J2315. Each fraction was added to 10ml of ISB containing 1×10^5 cfu/ml J2315 and incubated overnight at 37°C with shaking. 100µl dilutions were spread onto nutrient agar and the plates incubated for 48 hours 37°C to assess numbers of remaining viable bacteria.

2.2.12 Electron Microscopy

Transmission Electron Microscopy

Bacterial cells were fixed in 0.4% formaldehyde and the preparations centrifuged at 100 000g for 1 hour to deposit cells or cell debris. After centrifugation, the pellet was then gently resuspended in 1% w/v ammonium acetate buffer. The samples were then negative stained with 2% phosphotungstic acid and viewed using a CM12 Transmission Electron Microscope.

Scanning Electron Microscopy

Samples for scanning electron microscopy were prepared using the following method. Samples were fixed for three hours in 3% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.4, then washed three times in 0.1M sodium cacodylate

buffer for 20 minutes. The samples were then left in 0.1M sodium cacodylate buffer with 1% osmium tetroxide in for 2 hours, before washing in distilled water for 30 minutes. The samples were then dehydrated in increasing concentrations of acetone. First, at a concentration of 50% acetone for approximately 1 hour then at 70%, 90% and finally 100%, again for one hour. The 100% acetone step was then repeated twice before critical point drying with carbon dioxide was carried out in a Polaron E3000 SII CPD. The samples were then sputter coated with 20nm gold/palladium (60/40) in an EMscope SC500 sputter coater, and viewed in a Phillips 505 scanning electron microscope.

2.2.13 Activity of human and mouse β -defensin 1 against multiresistant *B. cepacia*

Experiments involving hBD-1 and mBD-1 were carried out in collaboration with Dr DJ Davidson, MRC Human Genetics Unit, Western General Hospital.

The antimicrobial activity of human and mouse salt-sensitive β -defensins was investigated in the presence of various concentrations of NaCl. Experiments were carried out in 0.01M phosphate buffer which contained 8.02ml of 1M K_2HPO_4 , 1.98ml of 1M KH_2PO_4 and 1 gram of D-Glucose in 1000ml distilled H_2O . 10 x stock solutions of sodium chloride were prepared as shown in Table 2.3. A 5 x stock solution of the peptide to be tested was then prepared by dissolving approximately 300 μ g of peptide in 600 μ l phosphate buffer. For each concentration of salt, two peptide containing reaction mixes and two controls were generated. The reaction mix contained 50 μ l of 5x β -defensin 1, 47.5 μ l of the appropriate 10 x salt solution, 25 μ l of a 5×10^7 cfu/ml suspension of bacteria and 377.5 μ l of 0.01M phosphate

buffer. Controls contained the same components as the reaction mix except the 50µl of peptide was replaced with the same volume of phosphate buffer.

The bacterial inoculum was prepared from an overnight culture *B. cepacia* (J2315) containing approximately 1×10^9 cfu/ml. Bacteria were centrifuged at 4000 x g for 10 minutes and the supernate discarded and the bacterial pellet re-suspended in PBS. A 1ml aliquot of this bacterial suspension was then prepared for each salt concentration. The 1ml aliquots of bacteria were spun down at 4000 x g in Sorvall centrifuge (Stevenage, Herts, UK) at room temperature for 10 minutes, and the bacteria resuspended in 10 ml of 1 x salt buffer.

This suspension was then diluted to a concentration of 5×10^7 cfu/ml and 25µl of this added to the peptide reaction mixes and blank controls.

Each tube was mixed thoroughly and incubated at 37°C for 25 minutes. After this time 10^1 and 10^2 dilutions were made of each sample and spread onto plates of CIA. Plate cultures were then incubated at 37°C for 48 hours and the number of cfus assessed.

Table 2.3 10 x stock solution of NaCl

<u>NaCl mM</u>	<u>10ml</u>
0	0
30	0.175g
60	0.351g
90	0.526g
120	0.701g
150	0.877g

2.2.14 Investigation of the antimicrobial activity of elafin

Samples of elafin, a proteinase inhibitor produced in the human lung, were provided by Dr J-M. Sallenave, Rayne Laboratory, University of Edinburgh Medical School. They were prepared by transfecting the human cell line A549 with adenovirus containing the elafin gene causing the cells to over express this protein (Sallenave et al, 1998). Elafin samples were dialysed extensively with water and freeze dried before being tested.

Cultures of strains of *S. aureus* (C1705), *P. aeruginosa* (PAO1) and *B. cepacia* (J2315) were grown up in 10ml of nutrient broth plus 5% yeast extract, overnight at 37°C in an orbital incubator (Gallenkamp). 100µl of the overnight cultures was inoculated in fresh bottles of nutrient broth and incubated at 37°C for three hours, until the bacteria were in log growth phase as concluded from predetermined growth curves.

The log phase bacterial cultures were spun down at 4000 x g for 20 minutes and the supernatant removed and replaced with 10ml of 0.01M phosphate buffer. This step was then repeated to insure all the nutrient broth had been removed. Bacterial suspensions were then diluted in phosphate buffer to a concentration of approximately 5×10^4 colony-forming units per millilitre (cfu/ml).

50µl of this concentration of bacteria were then placed into the microcentrifuge tubes containing adeno-elafin, mixed thoroughly and incubated for 2 hours at 37°C.

After this incubation, a neat sample and a 1 in 10 dilution from each tube were spread onto plates of suitable agar and incubated for 24-48 hours until colonies were visible and viable counts carried out.

CHAPTER 3 RESISTANCE OF *B. CEPACIA*

3.1 Description of strains included in the *B. cepacia* strain panel

The panel of 20 '*Burkholderia cepacia*' isolates used throughout this thesis was selected to include clinical and environmental strains (Table 2.1). As this thesis progressed, the important taxonomic data from Peter VanDamme was published, (University of Gent) clearly showing that isolates tentatively identified as *B. cepacia*, belonged to several individual subpopulations showing close phenotypic and genomic relationships. The term *B. cepacia* complex is sometimes used to describe all the members of this rapidly expanding genus. The strain panel was constituted to include the range of *B. cepacia* 'genomovars' known at the time and for the purpose of this thesis the term *B. cepacia* will be used throughout.

Fourteen clinical isolates collected from CF and non-CF patients included epidemic strains responsible for large outbreaks in CF centres in Edinburgh, Glasgow, Newcastle and Manchester. Non-CF isolates consisted of two from the American Type Culture Collection (ATCC 17765 and ATCC 25608), another from a cerebral abscess which occurred in an otherwise healthy individual following treatment of an ear infection with antibiotic drops contaminated with *B. cepacia*, and finally one non-CF isolate was from a patient with septicaemia. The five environmental isolates selected included two strains cultured from soil collected in the Royal Botanical Gardens, Edinburgh, three ATCC strains (ATCC 10856, ATCC 17516 and ATCC 25416) and AMMD, a strain developed for use as a biological control agent.

As a first step, pulsed field gel electrophoresis following digestion of chromosomal DNA with the restriction enzyme XbaI was used to confirm that the collection of isolates represented individual strains of *B. cepacia* and to confirm the clonality of *B. cepacia* C1963 (non-mucoid) and C1964 (mucoid). It can be observed from Figures 3.1 and 3.2 that 17 of the isolates analysed possessed unique banding patterns and thus can be described as individual strains of *B. cepacia*. Despite repeated attempts, no banding pattern could be obtained for isolate J2552. This phenomenon has been observed previously in *B. cepacia* and attributed to high levels of endogenous DNase (C. Doherty, personal communication). For the purpose of this thesis, J2552 was assumed to be clonally unrelated to other isolates. As expected, the non-mucoid (C1963) and mucoid (C1964) colonial morphotypes isolated from the same patient produced an identical PFGE profile. Two isolates, C1632 and C1773, for which no epidemiological connection could be determined, produced PFGE profiles differing by only four bands. Since this is on the borderline of what is acceptable to differentiate isolates by PFGE, these isolates were retained in the study as distinct strains.

Fig. 3.1 PFGE patterns of isolates of *B. cepacia*. Lanes 1-10; C1052, C1511, C1576, C1632, C1773, C1962, C1963, C1964, J415 and J419 respectively.

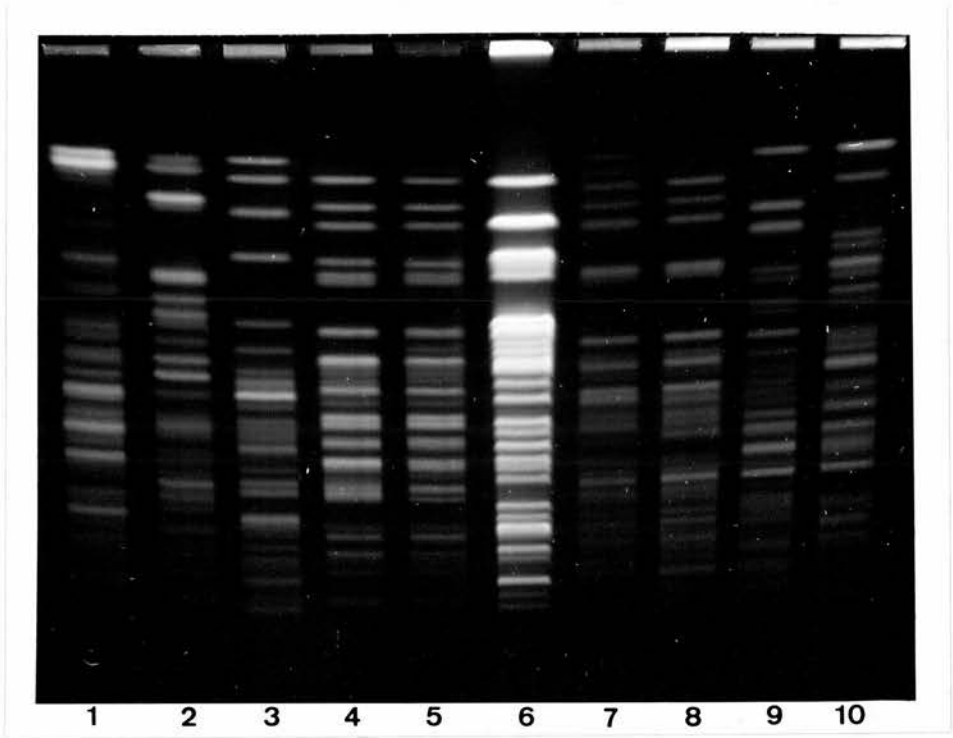
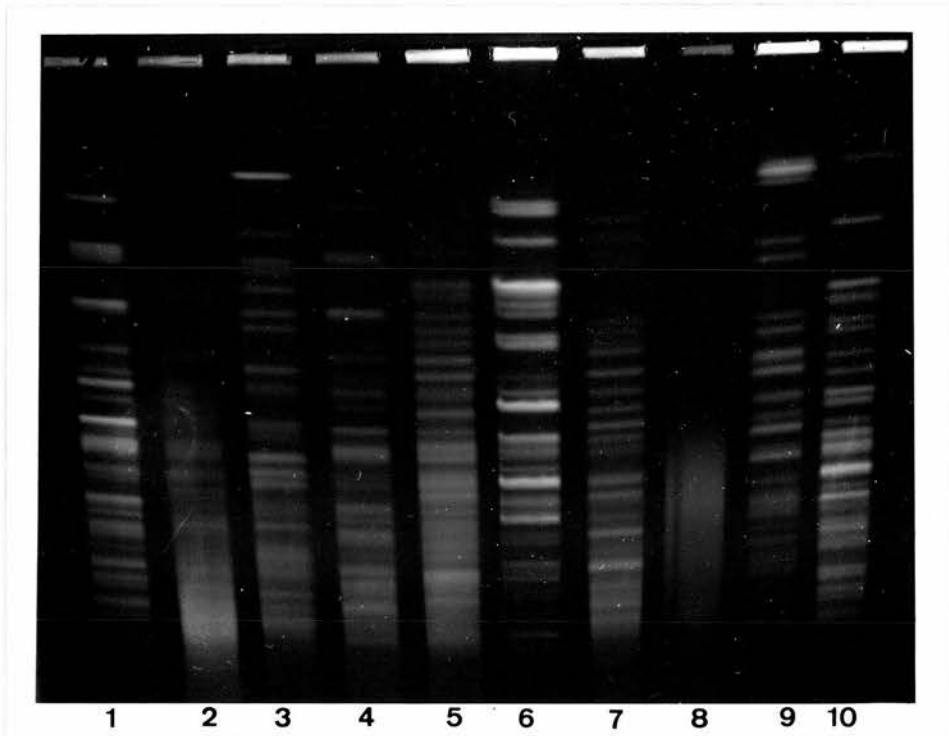


Fig. 3.2 PFGE patterns of isolates of *B. cepacia*. Lanes 1-10; J673, J2315, J2421, J2502, J2503, J2524, J2537, J2552, J2742 and A507 respectively.



3.2 Susceptibility testing

The sensitivity of the panel of *B. cepacia* isolates to conventional antibiotics was determined by both the broth macrodilution method employing ISB and by the agar dilution method employing ISA according to standard guidelines (Phillips et al, 1991). Breakpoint information is also provided within these standard guidelines. MIC values are outlined in Tables 3.1 and 3.2.

Despite its reputation as a pan resistant species, with the exception of colomycin, polymyxin and vancomycin, a wide range of inhibitory activity was observed against the *B. cepacia* panel. Indeed, for several antibiotics including chloramphenicol, trimethoprim and rifampicin some members of the *B. cepacia* panel were found to be more susceptible than the *P. aeruginosa* control strain NCTC 10662. In terms of antibacterial activity ciprofloxacin, grepafloxacin and meropenem appeared to be the most active, and polymyxin the least active.

Table 3.1 MICs of conventional antibiotics against the *B. cepacia* panel measured by broth macrodilution method.

Strain	Ceftaz	Mero	Chlor	Cipro	Grepa	Trimeth	Rifamp	Tobra	Colo	Poly	Vanco
C1052	4	4	8	1	0.25	1	8	32	>256	>512	>256
C1511	4	2	>32	8	1	32	>32	>32	>256	>512	>256
C1576	>32	8	>32	16	8	4	>32	>32	>256	>512	>256
C1632	>32	8	>32	16	1	8	>32	>32	>256	>512	>256
C1773	32	0.5	8	>32	32	1	16	32	>256	512	256
C1962	1	0.5	8	8	2	4	8	32	>256	>512	>256
C1963	1	0.5	32	2	0.5	4	16	>32	>256	>512	>256
C1964	1	0.25	32	2	0.5	1	16	4	>256	>512	>256
J415	2	0.5	>32	8	2	32	>32	>32	>256	>512	>256
J419	1	1	>32	>32	>32	>32	>32	32	>256	>512	>256
J673	2	1	16	0.5	0.25	1	32	2	>256	>512	>256
J2315	16	16	32	8	2	32	>32	>32	>256	>512	>256
J2421	4	4	16	4	1	2	32	16	>256	256	>256
J2502	0.25	1	16	0.5	0.25	1	8	2	>256	>512	>256
J2503	2	1	32	4	4	1	16		>256	>512	>256
J2524	4	2	>32	2	0.25	4	>32	32	>256	>512	>256
J2537	2	2	16	4	2	2	32	>32	>256	>512	>256
J2552	1	1	8	1	0.5	1	8	8	>256	512	>256
J2742	0.25	0.5	8	0.5		1	8	4	>256	>512	>256
A507	1	0.5	>32		1	32	>32	32	>256	>512	32
MIC50	2	1	32	4	1	2	32	32	>256	>512	>256
MIC90	32	8	>32	16	8	32	>32	>32	>256	>512	>256
Break-point	2	4	8	1	1	0.5	1	1	4	4	4
NCTC 10662*	2	1	>32	0.5	0.25	32	32	0.12	8	0.5	>256

* *P. aeruginosa* control

Table 3.2 MICs of conventional antibiotics against the *B. cepacia* panel measured by agar dilution method.

Strain	Ceftaz	Mero	Chlor	Cipro	Grepa	Trimeth	Rifamp	Tobra	Colo	Poly	Vanco
C1052	2	4	4	0.5	0.12	0.5	8	16	>256	512	>256
C1511	4	1	32	2	1	16	>32	>32	>256	>512	>256
C1576	>32	2	32	4	4	4	>32	>32	>256	>512	>256
C1632	>32	4	32	4	0.5	4	>32	>32	>256	>512	>256
C1773	16	1	8	32	32	1	8	>32	>256	512	>256
C1962	0.5	0.12	2	2	1	2	4	32	>256	>512	>256
C1963	1	1	16	1	0.5	32	16	32	>256	>512	>256
C1964	0.5	0.5	32	1	0.5	1	32	1	>256	>512	>256
J415	1	0.5	32	4	2	16	>32	>32	>256	>512	>256
J419	1	0.5	32	>32	32	32	>32	32	>256	>512	>256
J673	1	0.5	16	0.5	0.25	0.5	16	16	>256	>512	>256
J2315	4	4	32	4	2	32	>32	>32	>256	>512	>256
J2421	4	2	16	1	0.5	1	32	8	>256	256	>256
J2502	0.12	0.12	8	0.5	0.12	0.5	4	16	>256	>512	128
J2503	2	0.5	16	4	2	0.5	16		>256	>512	>256
J2524	1	1	16	1	0.25	2	16	>32	>256	>512	>256
J2537	1	0.5	16	2	2	1	16	32	>256	>512	>256
J2552	1	0.5	8	1	1	0.25	16	4	>256	>512	>256
J2742	0.5	0.25	8	0.25		0.5	4	8	>256	>512	>256
A507	1	0.25	>32		1	32	>32	32	>256	>512	64
MIC50	1	0.5	16	2	1	1	16	32	>256	>512	>256
MIC90	16	2	32	32	32	32	>32	>32	>256	>512	>256
Break-point	2	4	8	1	1	0.5	1	1	4	4	4
NCTC 10662*	1	0.25	>32	0.25	0.12	32	32	0.25	8	0.5	>256

* *P. aeruginosa* control

3.3 Analysis of resistance genes

To investigate the presence of known resistance mechanisms in the strain panel, primers were used to identify a) the penicillinase gene *penA*, first identified in the strain J2421 (249) (Trepanier et al, 1997), b) *opcM* which codes for an efflux pump originally identified in strain C3055 (K61-3) (Burns et al, 1996b). Preparations of DNA were made for all 20 *B. cepacia* isolates. Amplification of small sequences of this DNA containing sections of the antibiotic resistance genes was carried out by PCR and the amplified fragments visualised by agarose gel electrophoresis. Interestingly, although the presence of *penA* was confirmed in the *B. cepacia* control strain J2421, the gene sequence was not detected in any of the other 19 isolates of *B. cepacia* analysed (Fig 3.3). In contrast figure 3.4 confirms the presence of *opcM* in the control strain C3055 and in almost all the other 20 *B. cepacia* isolates analysed. An identical sequence was also amplified from a *P. aeruginosa* control.

Fig 3.3 PCR amplification of sequence of the *penA* gene. Lanes 1 and 22 contain a 100bp marker. Lanes 2-21 contain C1052, C1511, C1576, C1632, C1773, C1962, C1963, C1964, J415, J419, J673, J2315, J2421, J2502, J2503, J2524, J2537, J2552, J2742 and A507 respectively. Lane 23 is the negative control.

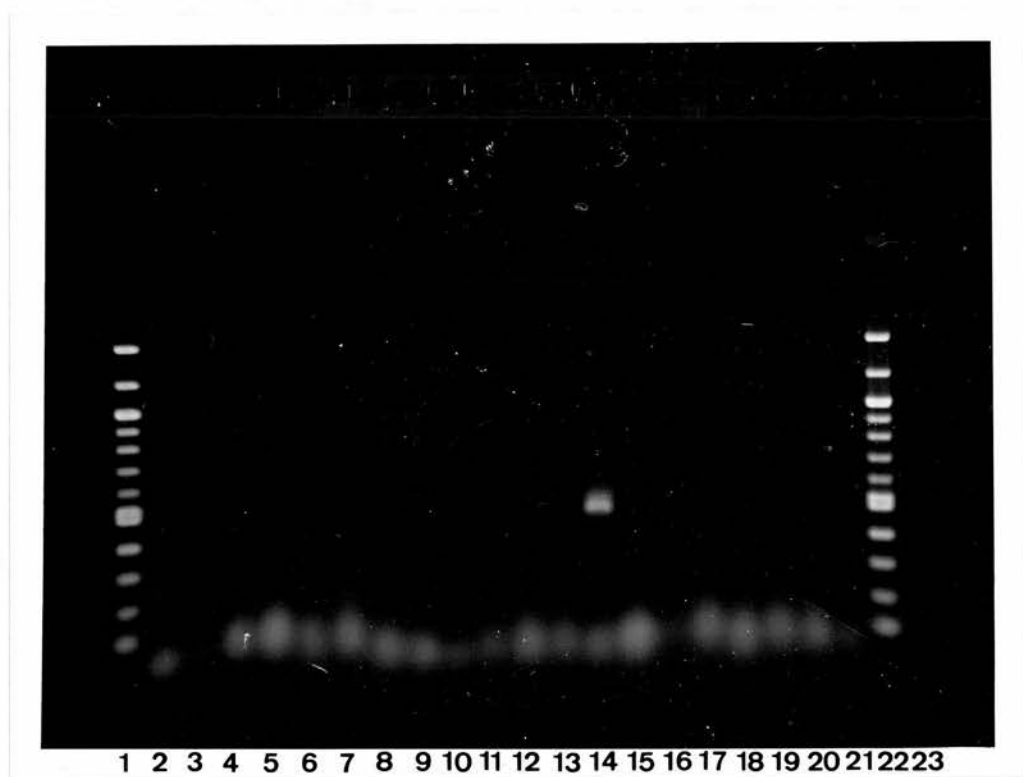
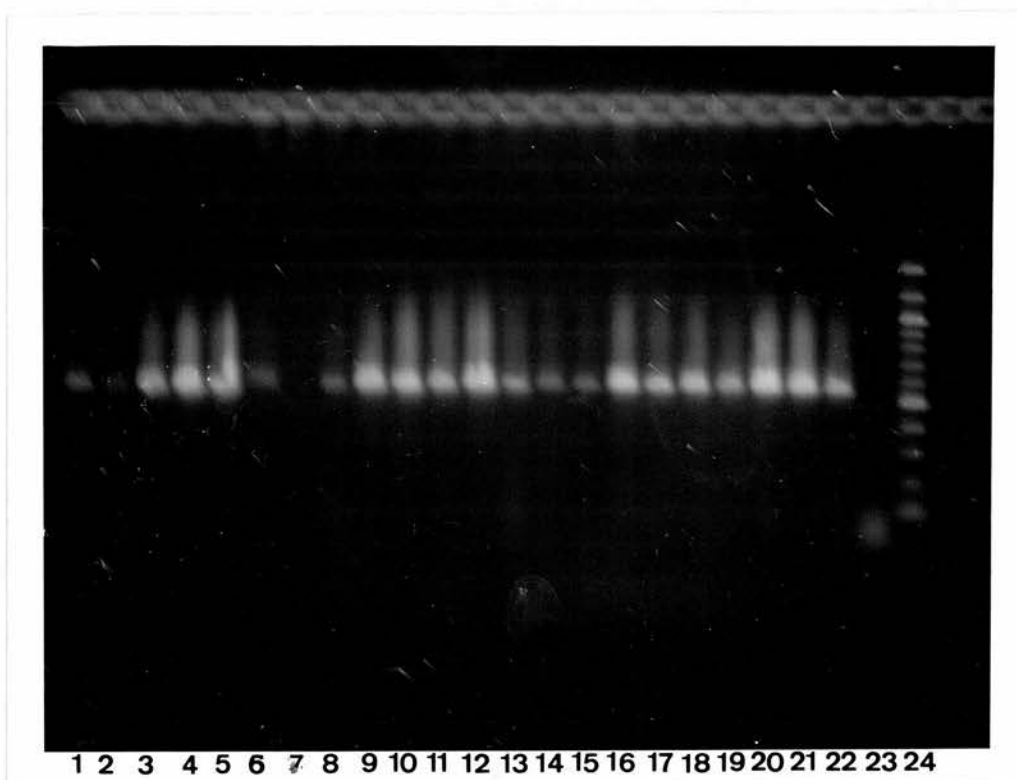


Fig 3.4 PCR amplification of sequence of the *opcM* gene. Lanes 1-22 contain C1052, C1511, C1576, C1632, C1773, C1962, C1963, C1964, J415, J419, J673, J2315, J2421, J2502, J2503, J2524, J2537, J2552, J2742, A507, C3055 and NCTC 10662. Lane 23 contains a negative control. Lane 24 contains a 100bp marker.



3.4 Discussion: Resistance of *B. cepacia*.

3.4.1 Susceptibilities to conventional antibiotics

The chemotherapeutic agents used in this study were selected to represent the major classes of antibiotics including conventional antipseudomonal agents representing standard care for *B. cepacia* infections. These agents inhibit bacterial growth by disrupting different stages of the cell cycle unique to bacteria. The β -lactams, ceftazidime and meropenem inhibit bacterial cell wall synthesis. Ceftazidime, a third generation cephalosporin and meropenem, a carbapenem are also amongst the most active agents against *P. aeruginosa* and have been reported to have useful activity against *B. cepacia* strains in vitro (Lewin et al, 1993; Pitt et al, 1996). Vancomycin, a glycopeptide, is also an inhibitor of cell wall synthesis but has a narrow spectrum of activity which principally involves Gram-positive organisms. Vancomycin was included in this study for interest due to the unpredictability of *B. cepacia* and the inherent activity of the agent. Tobramycin, an aminoglycoside antibiotic is selectively active against prokaryote 70s ribosomes and thus disrupts bacterial protein synthesis. Nebulised tobramycin is presently undergoing major clinical trials in the treatment of *P. aeruginosa* lung infections in CF patients and results are encouraging (Ramsey et al, 1999). Ciprofloxacin and grepafloxacin are fluoroquinolones whose bactericidal activity is principally mediated by interference with DNA gyrase and thus inhibition of bacterial DNA synthesis. Both agents have a broad spectrum activity. Grepafloxacin, the most recently developed quinolone, is considered to have enhanced activity against Gram-positive organisms whilst retaining good activity against Gram-negative and atypical organisms (Imada et al,

1992). Previous reports have found most strains of *B. cepacia*, particularly those isolated from clinical environments, to be resistant to ciprofloxacin (Lewin et al, 1993; Pitt et al, 1996; Visalli et al, 1997).

Historically, trimethoprim, an inhibitor of folate synthesis, and chloramphenicol, an inhibitor of bacterial protein synthesis, were reported to be the most effective antibiotics for treatment of *B. cepacia* infections in CF patients (Isles et al, 1984) (Lewin et al, 1993). However, these agents were rarely capable of eradicating *B. cepacia* from the respiratory tract of patients (Isles et al, 1984). More recently developed agents such as ceftazidime and meropenem appear to have superseded trimethoprim and chloramphenicol as the agents of choice against *B. cepacia*. The antimicrobial peptides, colomycin and polymyxin, were included in this study as increased use of these drugs during the 1980's provides an attractive hypothesis to explain the selection of *B. cepacia* in the CF lung and the striking increase in prevalence of *B. cepacia* infections during the last two decades (Littlewood et al, 1985). Previous studies have shown all isolates of *B. cepacia* to be resistant to colomycin and polymyxin (Ferreira et al, 1985; Moore and Hancock, 1986). Rifampicin was included in this study since it is the only clinically important antibiotic to inhibit bacterial RNA synthesis.

To assess the effect of different methods of susceptibility testing for *B. cepacia*, MICs of each of the antimicrobial agents were assayed against the panel by both the broth macrodilution method and the agar dilution method. Results indicated that the outcomes are generally concordant, or one dilution lower by the agar dilution method. For the purpose of this thesis, the broth method was chosen for further studies as it provides a more consistent inoculum and more even distribution of the

antibacterial agent through the culture media. This factor was particularly relevant in the investigation of the antimicrobial activity of plant oils which are more difficult to disperse (Hammer et al, 1999).

Although the panel comprised a relatively small number of *B. cepacia* isolates, it was interesting to note differences in susceptibility between environmental and clinical isolates and, taxonomically, differences between members of the *B. cepacia* complex. In general, the environmental isolates proved to be more sensitive to antibiotics than clinical isolates. For example, five of the six environmental isolates were sensitive to ceftazidime, in contrast only half of the clinical isolates were sensitive to this agent. All three meropenem resistant strains were clinical isolates whereas all of the environmental isolates were sensitive. Only one environmental strain was resistant to grepafloxacin whereas half of the clinical strains were resistant. Also two thirds of the environmental isolates were sensitive to ciprofloxacin, but only one clinical isolate was sensitive. Interestingly, most strains were resistant to chloramphenicol and all of the strains tested were resistant to polymyxin, rifampicin, trimethoprim, tobramycin and vancomycin.

Genomovar III strains were found to be particularly resistant to the antibiotics investigated. Recently, isolates of *B. cepacia* classified as genomovar III have been identified in the environment (Govan et al, 2000). Isolates studied in this thesis were selected before environmental genomovar III isolates were discovered, thus, the nine genomovar III strains investigated in this thesis were all clinical isolates. Two of the three strains resistant to meropenem were genomovar III strains. More than half of these strains were resistant to ceftazidime or grepafloxacin, which is a higher proportion than observed for clinical strains as a whole. Only one genomovar III

strain was sensitive to chloramphenicol and none sensitive to ciprofloxacin.

Genomovar II isolates were also observed to have a broader resistance spectrum than genomovar I isolates. Of particular interest was the observation that the only environmental genomovar II strain was resistant to all the antibiotics tested except meropenem and grepafloxacin.

Only two of the twenty isolates tested were completely resistant to all of the antibiotics. These were C1576, representing the Glasgow epidemic strain (genomovar II) and J2315, representing the Edinburgh epidemic strain and ET12 lineage (genomovar III). The other epidemic strains included in this study were also highly resistant to most antibiotics. C1632, the Newcastle epidemic strain was sensitive only to grepafloxacin and the Manchester epidemic strain, C1511, was sensitive only to grepafloxacin and meropenem.

Thus, with the caveat that the panel represents a relatively small number of *B. cepacia* isolates, it appears that, in this study, clinical isolates are relatively more resistant to antibiotics than environmental isolates. From a taxonomic viewpoint, the genomovars II and III isolates were the most resistant. It should be noted, however that not all genomovars were represented in this study. The four epidemic strains were observed to be particularly resistant to the antibiotics tested.

Table 3.3 Comparison of sensitive and resistant isolates of *B. cepacia*

	ceftaz	mero	chlor	cipro	grepa	trimeth	rifamp	tobra
Clinical								
I								
C1963	S	S	R	R	S	R	R	R
C1964	S	S	R	R	S	R	R	R
II								
C1052	R	S	S	S	S	R	R	R
C1576	R	R	R	R	R	R	R	R
C1962	S	S	S	R	R	R	R	R
III								
C1511	R	S	R	R	S	R	R	R
C1632	R	R	R	R	S	R	R	R
C1773	R	S	S	R	R	R	R	R
J415	S	S	R	R	R	R	R	R
J419	S	S	R	R	R	R	R	R
J2315	R	R	R	R	R	R	R	R
J2503	S	S	R	R	R	R	R	
J2524	R	S	R	R	S	R	R	R
A507	S	S	R		S	R	R	R
Environmental								
I								
J2552	S	S	S	S	S	R	R	R
II								
J2421	R	S	R	R	S	R	R	R
V								
J2742	S	S	S	S		R	R	R
I-III-IV								
J673	S	S	R	S	S	R	R	R
J2502	S	S	R	S	S	R	R	R
J2537	S	S	R	R	R	R	R	R

3.4.2 Analysis of resistance genes of *B. cepacia*

The *pen A* gene of *B. cepacia* 249 (J2421) encodes an inducible class A β -lactamase (Trepanier et al, 1997). Amplification of a small sequence (493bp) of this resistance gene by PCR and subsequent visualisation of this fragment by agarose gel electrophoresis confirmed that this gene sequence was present in the control strain J2421. Rather surprisingly, this sequence was not amplified in any of the other *B. cepacia* isolates tested. Thus it appears that none of the other 19 *B. cepacia* isolates contain an identical DNA sequence. As ceftazidime resistance is widespread among *B. cepacia* isolates, it is surprising that Pen A appears to be a rare enzyme in strains of *B. cepacia* other than J2421. It is possible that a gene similar to *pen A* is present in other strains of *B. cepacia* but that the primers chosen match a sequence which is not conserved. However, particularly in view of the genetic diversity displayed by members of the *B. cepacia* complex, it seems more likely that a range of very different β -lactamases are encoded by different *B. cepacia* strains.

OpcM, a gene subcloned from *B. cepacia* strain K61-3 (C3055) codes for an outer membrane lipoprotein which is homologous to the *oprM* gene of *P. aeruginosa* (Burns et al 1996b). *OprM* codes for the outer membrane channel of a three part efflux pump present in *P. aeruginosa* known as MexA-MexB-OprM (Poole et al, 1993). Antimicrobial substrates eliminated from the cell by this pump include tetracycline, chloramphenicol, fluoroquinolones, β -lactams excluding carbapenems, novobiocin, erythromycin, fusidic acid and rifampicin (Li et al, 1995). The *oprM* efflux pump is one of three efflux pump systems also found in *P. aeruginosa*.

Following the recently completed sequencing of *P. aeruginosa* PAO1 it has been proposed that PAO1 contains a further 17 efflux pumps (Rick Gerber, personal

communication). In this thesis, amplification of a 564bp sequence of *opcM* by PCR and subsequent visualisation of this fragment by agarose gel electrophoresis confirmed that the sequence, detected by the primers, was present in the control strain of *B. cepacia* C3055 (K61-3), and demonstrated the presence of this pump in 19 *B. cepacia* isolates tested, and even in a *P. aeruginosa* control. On this evidence, it seems reasonable to speculate that *opcM* is highly conserved and present in most strains of *B. cepacia*. An important point for consideration is that although almost all the strains tested have the gene for this efflux pump, each has a different antibiogram. One explanation is that *opcM* is regulated by an as yet unknown resistance mechanism and that the protein OpcM is not produced in all isolates or that the number of channels produced varies from strain to strain. Furthermore, *B. cepacia* may utilise a number of other as yet undiscovered resistance mechanisms, capable of eliminating a variety of other substrates. As the primers also detected a 564bp sequence in the *P. aeruginosa* control, it seems likely that efflux systems of *B. cepacia* and *P. aeruginosa* are shared in some instances and have a conserved evolution. At present there are plans to sequence the entire *B. cepacia* genome. As this organism is 1.5 times the size of *P. aeruginosa* there is the potential that many unique gene sequences may be discovered which may encode additional resistance mechanisms.

CHAPTER 4 AQUEOUS GARLIC EXTRACT

4.1 Susceptibility testing with AGE

The panel of 20 *B. cepacia* strains was tested for susceptibility to AGE. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AGE for each isolate was determined using an inoculum of approximately 1×10^5 cfu/ml. A control for each isolate was prepared containing no AGE. In the controls, all inocula grew to concentrations of approximately 1×10^9 cfu/ml in the 18 hour incubation period; exceptions were the slower growing strains C1773 and J2513 which grew to concentrations of approximately 1×10^8 cfu/ml. Results are shown in Table 4.1. Overall the antibacterial activity of AGE against the *B. cepacia* panel showed a range of MIC's between 0.25% and 3%, with a MIC 90 of 2% and a range of MBC's between 3% and >6%, with an average of 5% recorded.

Table 4.1 MICs of Aqueous garlic extract

	MIC%	MBC%		MIC%	MBC%
A507	2	6	J673	3	>6
C1052	2	6	J2315	2	3
C1511	2	>6	J2421	1	>6
C1576	2	5	J2502	2	6
C1632	2	6	J2503	2	6
C1773	1	2	J2524	3	6
C1962	0.25	4	J2537	2	6
C1963	2	5	J2552	2	5
C1964	2	>6	J2742	2	3
J415	2	>6			
J419	2	5	NCTC10662	4	>6

The susceptibility of strains J2315 and J2421 to pure allicin preparation provided by L.D. Lawson was also tested. MICs were recorded of 0.024mg/ml and 0.08mg/ml respectively and MBCs of 0.08mg/ml and 0.12mg/ml respectively.

4.2 Killing curves

Log phase killing curves

The decline of logarithmic and stationary phase populations of clinical and environmental isolates of *B. cepacia* when challenged with 1 x MBC of AGE was studied. Kill curves for AGE against log phase cultures of a clinical isolate (J2315) and an environmental isolate (J2421) are shown in figures 4.1 and 4.2. Each experiment was carried out three times and on each occasion sampling was carried out in triplicate. Standard error of the mean error bars are shown.

The kill curve experiments showed that AGE takes nineteen hours to kill logarithmic phase cultures of J2315; in contrast, only nine hours was required to reduce the population of the environmental strain J2421 to almost zero. A small and transient recovery of growth of strain J2421 was observed at 10 hours.

Figure 4.1 The bactericidal action of AGE at 1 x MBC (3%) against a log phase cultures of *B. cepacia* J2315.

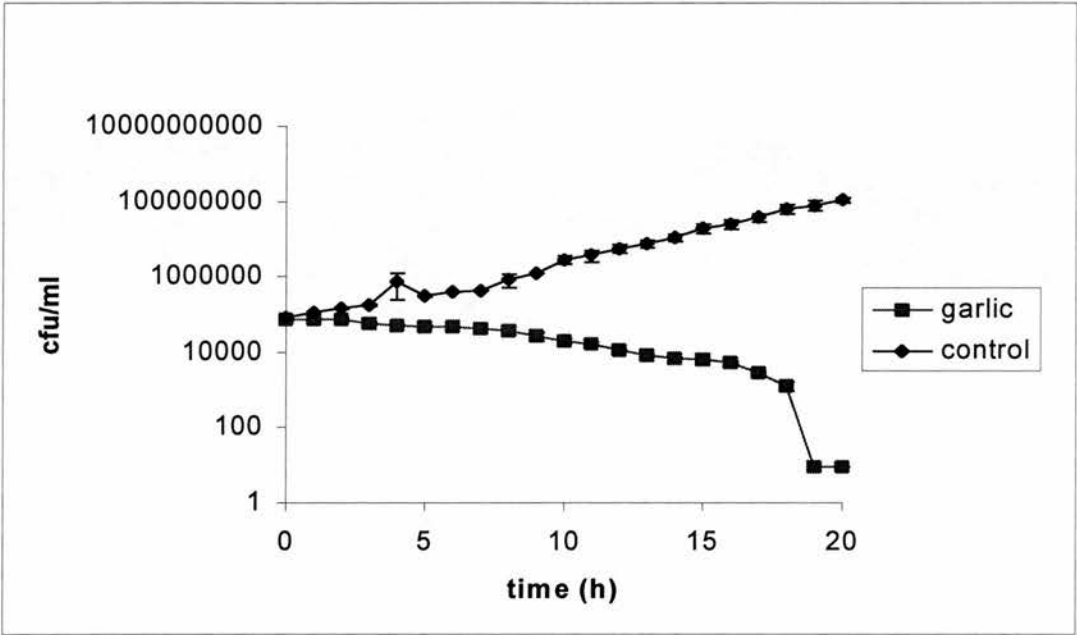
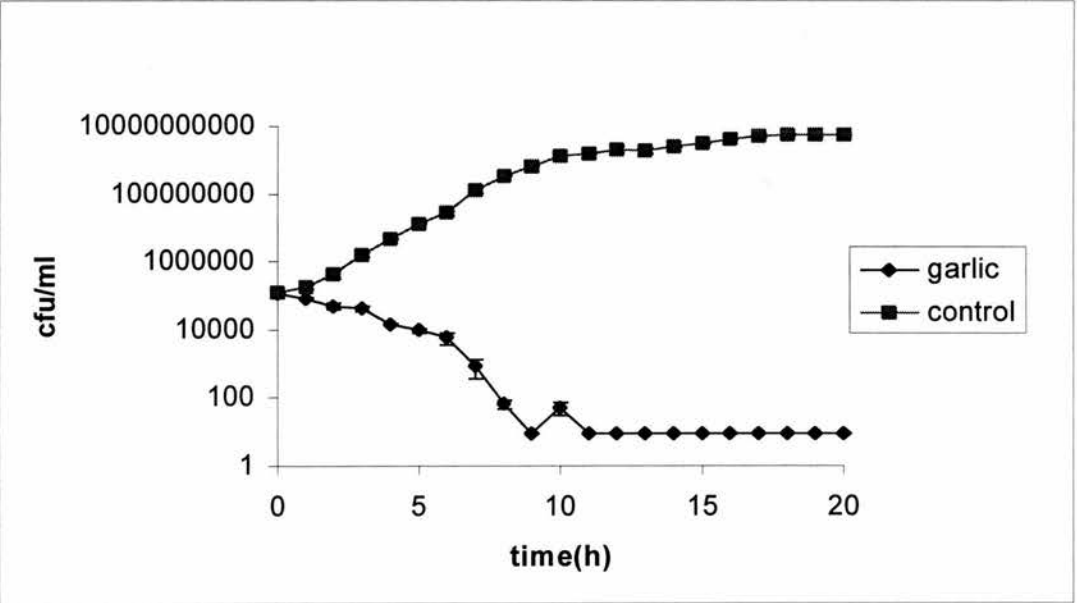


Figure 4.2 Bactericidal activity of AGE at 1 x MBC (6%) against a log phase culture of *B. cepacia* J2421.



Stationary phase killing curves

In further experiments, the bactericidal activity of AGE at 1 x MBC against stationary phase cultures of *B. cepacia* J2315, J2537, C1052 and A507, was measured at one hour intervals over a 20 hour period, and the results shown in figures 4.3, 4.4, 4.5 and 4.6.

The results showed that cfus present within of stationary phase cultures of the clinical strains, *B. cepacia* J2315, A507 and C1052 were considerably reduced by the MBC of AGE within 16 to 20 hours. A small recovery of growth was observed with strain J2315 after 17 hours. Initially, a similar pattern of inhibition was observed against the environmental strain J2537. After 12 hours, however, bactericidal activity against J2537 ceased and subsequently only bacteriostatic activity for the AGE was observed.

Figure 4.3 Bactericidal activity of AGE at 1 x MBC (6%) against a stationary phase culture of *B. cepacia* C1052.

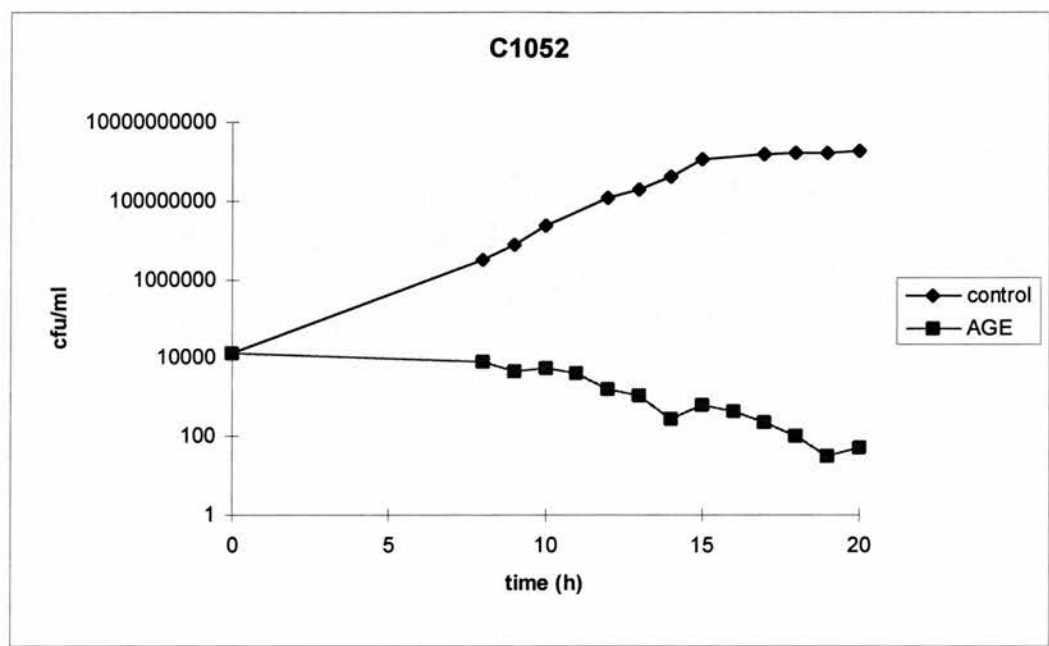


Figure 4.4 Bactericidal activity of AGE at 1 x MBC (6%) against a stationary phase culture of *B. cepacia* A507.

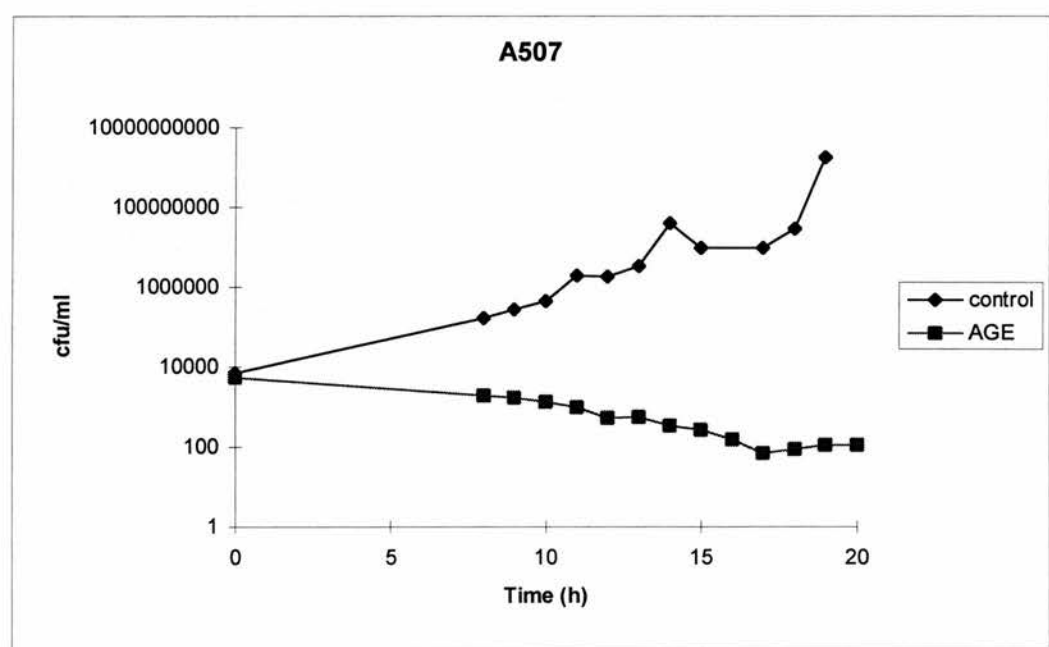


Figure 4.5 Bactericidal activity of AGE at 1 x MBC (3%) against a stationary phase culture of *B. cepacia* J2315.

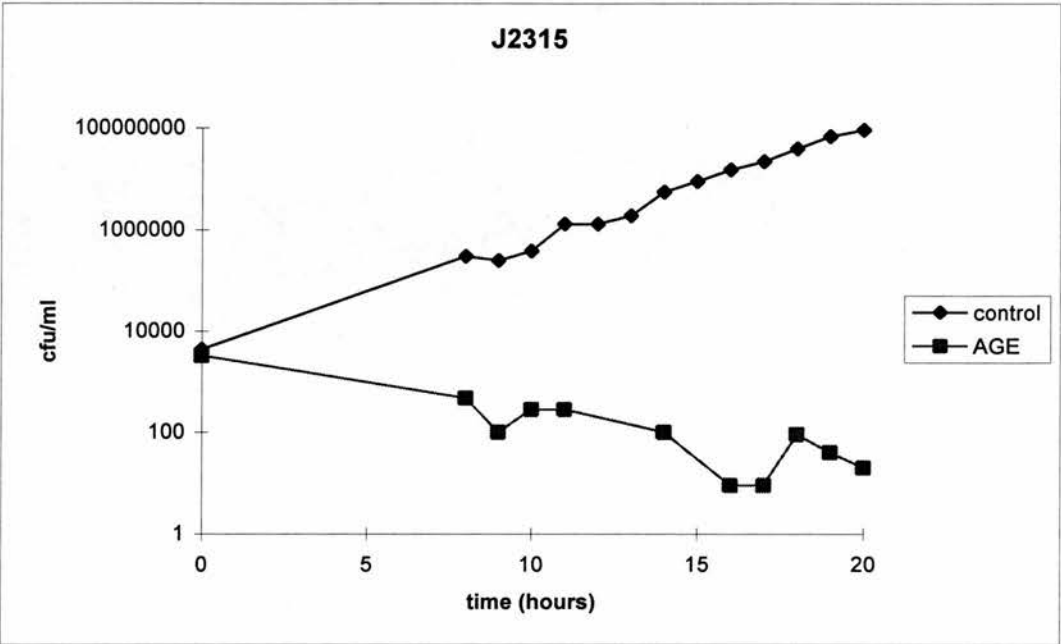
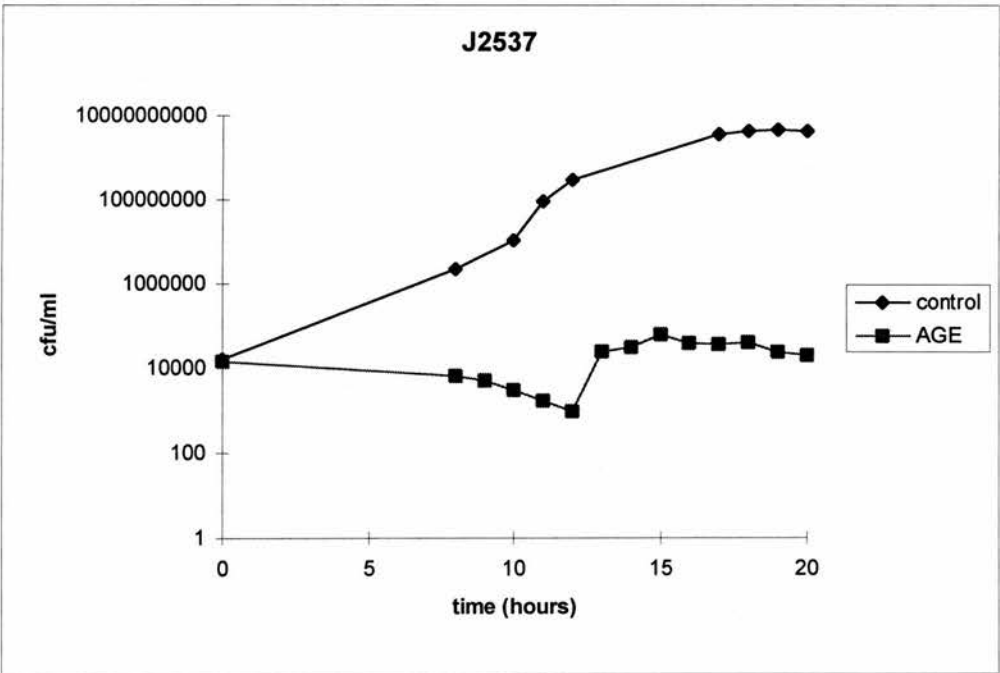


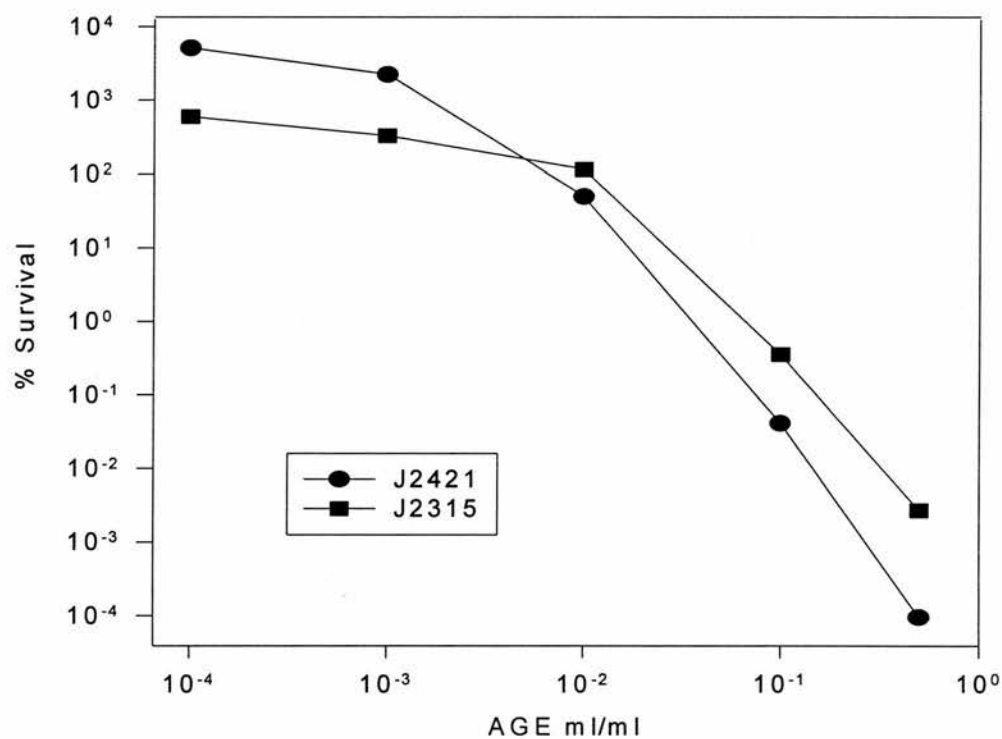
Figure 4.6 Activity of AGE at 1 x MBC (6%) against a stationary phase culture of *B. cepacia* J2537.



4.3 Dose response curves

The bactericidal activity of AGE against log phase cultures of *B. cepacia* J2315 and J2421 at a range of concentrations between 0.01% (v/v) to 50% (v/v) is shown in figure 4.7. Bacterial cfus were shown to decrease steadily with increasing concentrations of AGE.

Fig. 4.7 Survival of *B. cepacia* J2315 (initial concentration 3.36×10^5 cfu/ml) and J2421 (initial concentration 9.4×10^6 cfu/ml) after exposure to AGE for 5h and 4h respectively in iso-sensitest broth at 37°C.



4.4 Synergy testing by the checkerboard technique

To investigate the effects of AGE in the presence of conventional antibiotics six strains of *B. cepacia* (C1052, C1511, J419, J2315, J2524 and A507) from Table 2.1 were exposed to combinations of AGE and one of four antibiotics commonly used to treat CF lung infections. Isobolograms were produced for each strain with each combination of antimicrobials. Interestingly, with all of the six strains tested, most combinations of AGE and an antibiotic produced an antagonistic effect. However, synergy was observed with combinations of ceftazidime and AGE against strains J2315 and C1052 and the combination of AGE and ciprofloxacin produced synergistic activity against strains C1511 and A507. Combinations of chloramphenicol and AGE and meropenem and AGE produced only antagonistic effects against the six *B. cepacia* strains.

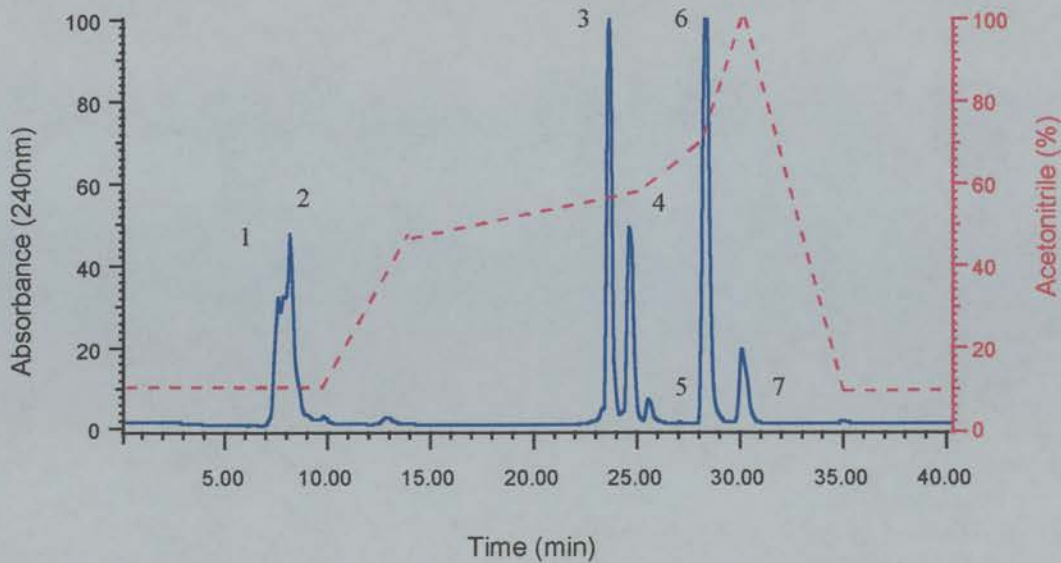
4.5 HPLC analysis of AGE

As spectrophotometry had revealed that allicin, the antibacterial agent within AGE absorbs light at a wavelength of 240nm; this wavelength was chosen to detect components of AGE separated by HPLC. AGE moieties were separated at 240nm on a gradient of acetonitrile in 0.1% (vol/vol) trifluoroacetic acid designed to give a clear separation of the constituents. Seven different compounds were detected in the sample. When this trace was compared with that of a sample of pure allicin, peak 7

was found to correspond to the peak produced when allicin was analysed under identical conditions. This suggested that peak 7 is the allicin fraction of AGE.

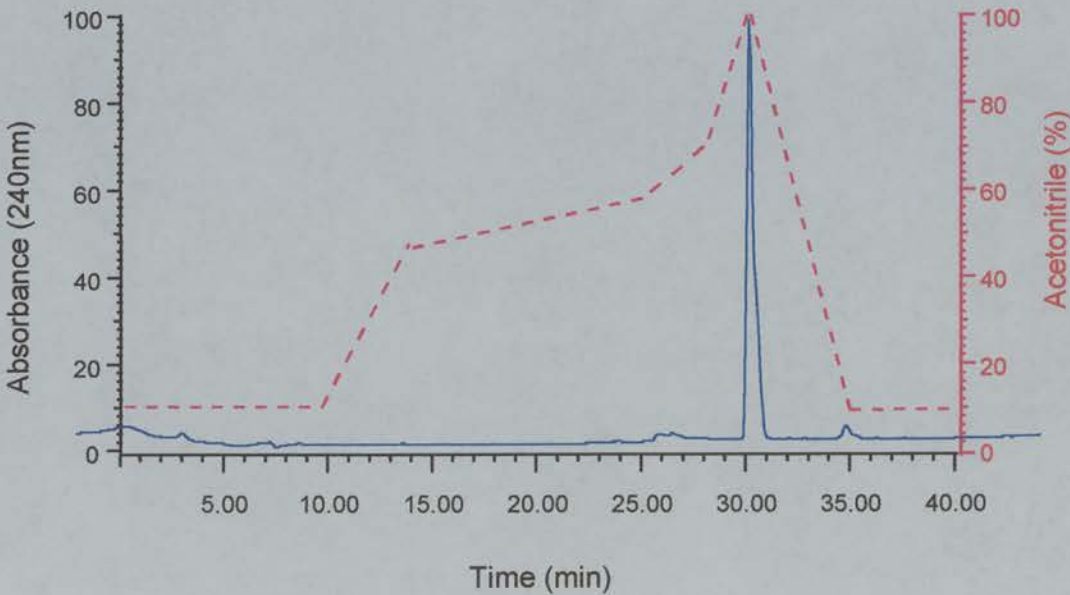
The fractions corresponding to these peaks were collected and spectrophotometry used to check that each sample only absorbed light at 240nm and thus contained no contaminants. Once the acetonitrile was removed by evaporation, these seven concentrated samples were reconstituted in 1ml ISB and tested for antimicrobial activity against *B. cepacia* strain J2315. Samples 3, 4, 6 and 7 each had considerable antimicrobial activity eradicating all bacteria present in the samples. Samples 1, 2 and 5 showed no notable antimicrobial activity. However, sample 5 may appear to have less antimicrobial activity as it is present at a considerably lower concentration than the other compounds.

Fig. 4.8 Separation of AGE by HPLC



The concentration of acetonitrile (solvent B) is shown by the broken line on the right hand axis.

Figure 4.9 Allicin HPLC trace



4.6 Electron Microscopy

Scanning and transmission electron microscopy revealed significant changes in the cellular structure of *B. cepacia* J2315 following two hours incubation in AGE. Apart from a reduction in the bacterial population compared to the control, the surviving bacteria were smaller than the control and their shape distorted.

Transmission electron microscopy

Figure 4.10 Control *B. cepacia* J2315

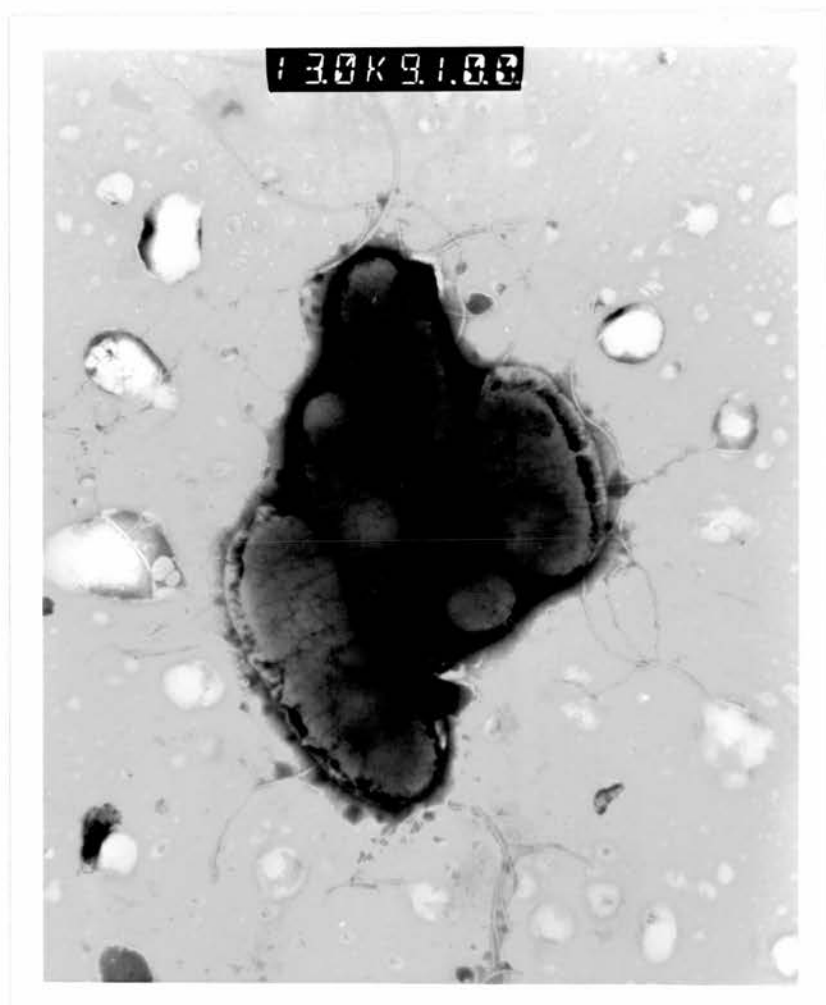


Figure 4.11 *B. cepacia* J2315 after two hours incubation with the 1 X MBC of AGE.

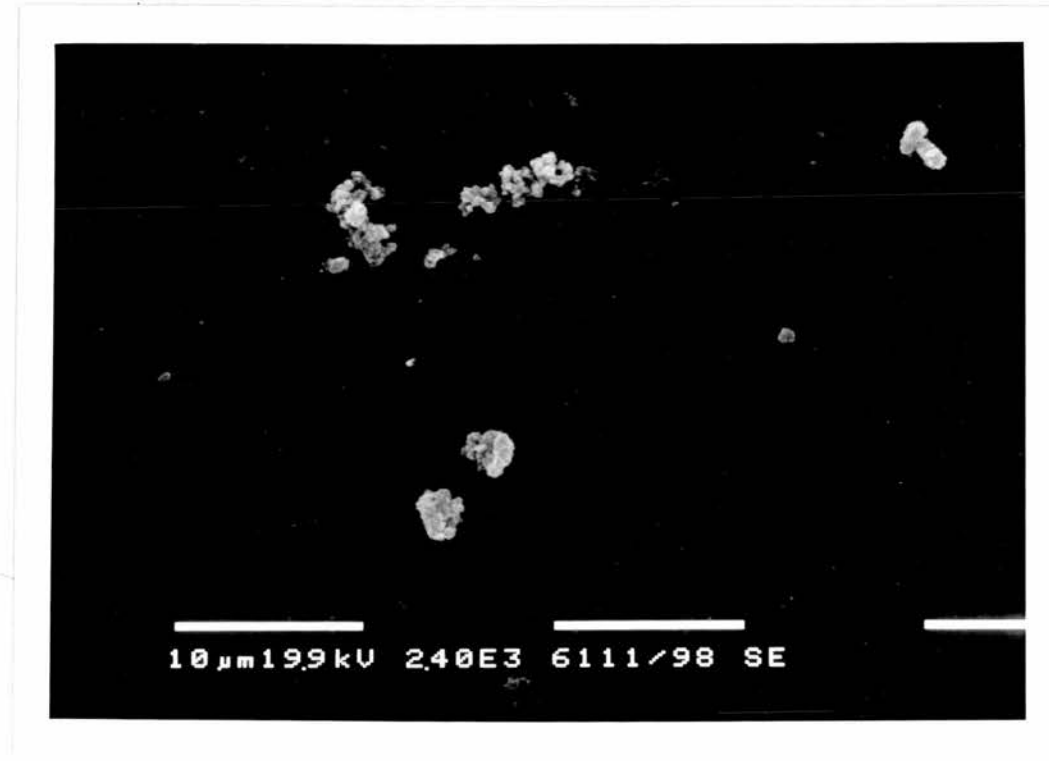


Scanning electron microscopy

Figure 4.12 Control *B. cepacia* J2315



Figure 4.13 *B. cepacia* J2315 after two hours incubation with the 1 X MBC of AGE.



4.7 Discussion: Aqueous Garlic Extract

Although garlic preparations in various forms are widely available in pharmacies and health food stores, many do not contain biologically active compounds because of the way they are prepared and stored. Freshly prepared aqueous extracts and certain dry powder preparations often contain detectable quantities of allicin and other thiosulphinates. However, only when partially chopped garlic cloves are dried at or below 60°C before grinding to garlic powder (Lawson and Hughes, 1992) or frozen in liquid nitrogen, pulverised and vacuum dried will extracts retain thiosulphinates and their precursor compounds. During storage humidity must be kept low to prevent mould formation and the destructive activation of alliinase (Lawson et al, 1991a). The amount of allicin and other thiosulphinates contained in commercial garlic powder has also been found to vary greatly between brands (Lawson et al, 1991a). As the quantity of thiosulphinates and storage history of these powders could not be guaranteed, it was decided to use freshly prepared aqueous extracts of garlic throughout this thesis. Aqueous garlic extracts contain similar amounts of allicin and other thiosulphinates to the best commercial garlic powder tablets (Lawson et al, 1991a). Foodstore-purchased garlic has been shown to contain more allicin and other thiosulphinates per gram than freshly picked garlic (Lawson et al, 1991a), therefore foodstore-purchased garlic was used to prepare AGE throughout this thesis. The antimicrobial activity of AGE was found to be stable at -70°C for one month.

4.7.1 Susceptibilities to AGE

Susceptibility of micro-organisms to garlic extracts has been the subject of considerable research. Important studies include those by Cavallito and Bailey (1944), which show that a 1 in 125 000 dilution of an allicin preparation was sufficient to inhibit the growth of a range of Gram positive and Gram negative bacteria.

Subsequent studies of aqueous and dried powder extracts of garlic include a study of 20 isolates of *Mycobacterium avium* complex, mainly from AIDS patients, that were inhibited by an AGE which contained 1 mg/ml of thiosulphinates (Deshpande et al, 1993). Cellini and co-workers (1996) observed that AGE at a concentration of between 2-5mg/ml was required to inhibit clinical isolates of *Helicobacter pylori*. Growth of *S. aureus* was shown to be completely inhibited after 24 hours incubation with 5% garlic powder extract (Gonzales-Fandos et al, 1994).

Other studies have shown that many bacterial species, including *Mycobacterium tuberculosis* and *Bacillus subtilis*, (Rao et al, 1946) *Bacillus cereus*, *Escherichia coli*, *Serratia marcescens* and *Pseudomonas aeruginosa*, (Elnima et al, 1983) *Shigella dysenteriae* (Al-Delaimy and Ali, 1970) and *Salmonella typhimurium*, (Johnson and Vaughn, 1969; Rees et al, 1993) are also inhibited by garlic extract concentrations of between 1 and 10%.

In this thesis, initial attention was directed towards the potential activity of AGE against a carefully selected panel of 20 *B. cepacia* isolates. Each of the isolates was also assessed for susceptibility to conventional antibiotics. The MIC 90 of AGE against the panel was 2% and the range of MBCs between 3% and >6%, with an average of 5%. These results correlate well with previous studies in which other

bacterial species were inhibited by similar concentrations of AGE. This concordance was surprising since *B. cepacia* is notorious for its inherent resistance to many conventional antibiotics.

When inhibitory activity of AGE against *B. cepacia* was compared with inhibition by conventional antibiotics, it became apparent that strains resistant to conventional antibiotics did not necessarily require the highest concentrations of AGE for inhibition and vice versa. For example, three highly resistant *B. cepacia* strains (C1962, C1773 and J2421) have MICs of less than 2% AGE.

Strains which exhibited the greatest resistance to AGE, *i.e.* MIC of 3% or MBC of more than 6% included the environmental isolates J673 and J2421 and the clinical isolates C1511, C1964, J415 and J2524. These strains do not show any pattern of resistance to conventional antibiotics. Also no particular genomovar is predominant amongst these strains.

In conclusion, because of the MIC data, it would appear that the mechanism of inhibition of AGE is unlikely to be shared with any the conventional antibiotics tested. The antibiotics included in this thesis were selected to represent each of the major classes of antibiotics which inhibit bacterial growth by disrupting unique points of the bacterial cell cycle. As the resistance profile of the strain panel to AGE is not comparable with that of any of the conventional antibiotics, we must conclude that AGE attacks bacterial cells by a mechanism not shared with conventional antibiotics.

4.7.2 Killing curves

Dose response curves show a constant decline in bacterial numbers with increasing concentrations of AGE. Thus, the MBC of AGE was used for all time / kill experiments.

The bactericidal activity of AGE is exemplified in the killing curves of log phase cultures of two *B. cepacia* strains. The multi-resistant ET12 isolate (J2315) is much slower growing than the environmental isolate J2421. J2315 did not reach a stationary phase of growth within the 20 hour period analysed whereas J2421 reached stationary phase after 11 hours. In the presence of AGE a slow decline in numbers of J2315 was observed over a period of 18 hours, until finally after 19 and 20 hours incubation, less than ten viable bacteria per millilitre were detected. The decline in bacterial numbers was much more pronounced for J2421. After nine hours less than ten cfu per millilitre were detected. There was a slight recovery after ten hours incubation, followed by an immediate decline.

J2315 and J2421 were originally isolated from different environments and have different antibiotic susceptibilities. J2315 is a multi-resistant clinical isolate whereas J2421 an environmental isolate is more sensitive to antibiotics. The difference in the rate of decline of these strains may indicate that strains isolated from the environment are more susceptible to AGE. Garlic plants produce the thiosulphinates present in AGE when they are damaged, as a defence mechanism to protect them from attack by micro-organisms. It would be of benefit to *Allium* species if plant pathogenic *B. cepacia* were highly susceptible to allicin and /or other thiosulphinates.

However, the environmental strain, J2421 required 6% AGE to be completely killed, double the concentration required to kill the clinical isolate J2315. The slower rate of killing observed with J2315 may exist solely because of the lower concentration of AGE used in that experiment. At concentrations of 6% AGE faster killing of J2315 may be observed.

When the log phase kill curves are compared with kill curves involving stationary phase cultures of *B. cepacia* it becomes apparent that J2315 has a slower rate of growth than the other strains studied, regardless of the stage of growth of bacteria used as the inoculum. When stationary phase cultures were challenged with AGE, a slow pattern of killing was displayed over a period of 16 – 20 hours for all the isolates. From the growth curve controls run alongside these killing experiments, it could be seen that cells from overnight cultures take approximately 15 hours to reach their stationary phase; the exception was J2315 which did not reach stationary phase within the 20 hour period studied. It can be seen from the growth curves in figures 4.1 and 4.2, in which log phase bacteria were used, that J2421 reached its stationary phase around nine to ten hours but once more J2315 did not reach stationary phase in the entire 20 hour period. Although J2315 is actually growing at log phase, its growth rate is much less than that of J2421. It appears that maximum killing of *B. cepacia* by AGE occurs as cells reach stationary phase. In conclusion, slower growing J2315 takes much longer to be killed than the other strains studied. There also may be a link between growth phase and killing by AGE.

Previously, Johnson and Vaughan (1969) studied killing of *S. typhimurium* and *E. coli* by dried garlic powder. Inhibition of *S. typhimurium* by pure allicin preparation has also been studied (Feldberg et al, 1988). The initial study carried out by Johnson and Vaughn showed that garlic powder was initially only bacteriostatic towards *S. typhimurium*, however bacterial killing was detected after two to six hours incubation with 10% garlic powder. This pattern was similar to that observed in this thesis with J2421. Thiosulphinates present in garlic powder other than allicin were also shown to have antibacterial activity against both resting and growing cells of *S. typhimurium*. N-propyl allyl disulfide and di-n-propyl disulfide were active against resting cells of *S. typhimurium*. However, these compounds exerted only a bacteriostatic influence against growing cells which was reversed in two to six hours (Johnson and Vaughn, 1969). In this thesis, unidentified compounds other than allicin, present within AGE have also been reported to inhibit *B. cepacia*. The possibility exists that thiosulphinates present within compete garlic extracts may act together to produce a greater bactericidal effect than would be observed for allicin alone.

The allicin content of the AGE used in this thesis could not be determined by the spectrophotometric method described by Han and co-workers (1995) as this method can only provide a measurement of total thiosulphinates present in a given sample. Therefore, MIC and MBC of pure allicin against strains J2315 and J2421 can not be compared with the MIC and MBC of AGE against these strains and, thus, the potential contribution of other thiosulphinates present within AGE to the anti-cepacia activity of this extract was not assessed.

Feldberg and co-workers also studied the effect of garlic compounds upon *S. typhimurium*, using a pure preparation of allicin. They proposed that allicin, at concentrations of between 0.2 and 0.5mM, was merely bacteriostatic and that inhibition was transient, lasting between 20 and 95 minutes. This inhibition period lengthened, with increased concentrations of allicin and with greater initial culture densities. Increasing concentrations of allicin also decreased the rate of growth, and when growth resumed after the inhibition period, the cell density at which stationary phase was entered, was lowered and the growth rate decreased.

It may be that if concentrations of allicin had been increased further, a bactericidal effect would have been observed. As the resumed growth rate was less than the original growth rate, it can be speculated that the cells do not totally recover from the effect of allicin inhibition and that some form of irreparable damage is achieved.

Thus, if greater concentrations of allicin were added greater lasting damage might be affected, and the cell recovery could be eliminated. It may also be that the discrepancy in the results of these two studies is due to the unstable nature of the garlic preparations used. Feldberg and co-workers used a relatively pure allicin preparation, whereas Johnson and Vaughn used a garlic powder, which contains many thiosulphinates including allicin that may contribute to the antibacterial effect. Synergy or an additive effect might also occur between the allicin and other antibacterial compounds known to be present in garlic.

4.7.3 Synergy studies

In this thesis, synergy testing carried out by the checkerboard technique described by Garrod and Waterworth (1962) revealed that combinations of AGE and conventional antibiotics were mostly antagonistic. Synergy was observed only with combinations of ciprofloxacin or ceftazidime with AGE and for only two *B. cepacia* strains. No additive combinations were observed. These results contrast with previous studies. Didry and co-workers tested combinations of garlic extract with ampicillin, doxycyclin, colistin, tobramycin, cefoxitin and thiamphenicol against eight bacterial strains selected to include both anaerobic and aerobic bacterial species. These did not include *B. cepacia*. No antagonism was observed. However, in approximately 50% of the combinations tested, synergy was observed, the most successful combinations involving aminoglycoside antibiotics and garlic extract (Didry et al, 1987; 1992). A separate study showed synergy in the combined action of streptomycin and allicin against tubercule bacilli (Gupta and Vaswanathan, 1955). The high incidence of antagonism in combinations of AGE with conventional antibiotics against *B. cepacia* may exist due to the presence of compounds in AGE not present in the garlic extract used by Didry and co-workers. In this thesis, no combination of AGE and an aminoglycoside was tested as *B. cepacia* is known to be inherently resistant to antibiotics of this class (Burns et al, 1989). There are no previous reports of the four antibiotics tested in this thesis (ceftazidime, ciprofloxacin, chloramphenicol and meropenem) being tested in combination with garlic against *B. cepacia* or other bacterial species. Thus, the high incidence of antagonism observed may be explained by the particular antibiotics used and the

strains tested. Overall the results obtained in this thesis appear to preclude the use of garlic extracts in combination with some conventional antibiotics commonly used for the treatment of *B. cepacia* infections.

4.7.4 HPLC

Thiosulphinates other than allicin were originally discovered and separated from garlic extracts by paper or thin layer chromatography (Barone and Tansey, 1977). These methods have been superseded as they lack the resolution, sensitivity and quantitation attainable by modern methods. Han et al (1995) defined a method useful for the quantification of total thiosulphinates present in a sample. This spectrophotometric method uses DTNB or DTDP to measure the decrease in cysteine concentration when allicin reacts with this cysteine to form S-allylmercaptocysteine. However, as other thiosulphinates also react with cysteine this method is not suitable for measuring allicin alone. Gas chromatography, a more advanced technique is also unsuitable as a means of separating allicin because of the high injection port and column temperatures are required. However, indirect analysis based on measuring the quantity of allicin degradation products present in a sample can be used (Lawson et al, 1991c). Analysis using normal phase HPLC of diethyl ether garlic extracts and garlic products was first reported in 1985 (Miething, 1985). This technique also proved unsatisfactory, as allicin is unstable in ether and in other solvents required. Reverse phase HPLC utilising 60% methanol / 40% water as the eluent, did not present these problems and its first successful use was reported by Jansen et al in 1987. Subsequent studies have used C₁₈ and Si reverse phase HPLC to identify and

quantitate all eight of the thiosulphinates present in a variety of garlic preparations (Lawson and Hughes, 1989; Lawson et al, 1991a;c). HPLC analysis has the advantage that it can be performed at room temperature, however the technique does require the use of an external standard. At present, HPLC is regarded as the best method available for the separation of thiosulphinates present in garlic preparations. In this thesis, reverse phase HPLC was employed using a Beckman (5µm pore size Ultrasphere ODS; internal dimensions - 150 by 10 mm) column. Detection was at 240nm and separation was performed using an acetonitrile / water gradient. The results were compared with an allicin standard. Seven compounds were collected from the separation and tested for antimicrobial activity. Only four of the compounds collected showed notable activity against *B. cepacia* and these may act together within AGE to inhibit *B. cepacia*. As the allicin standard was shown to contain only one compound it is unlikely that any decomposition of allicin took place during analysis. Thus, the trace produced can be used to tentatively identify the allicin fraction present in AGE separated under identical conditions. Separation of AGE revealed that compound seven is most likely to be allicin as it is eluted from the column at the same time and with the same concentration of acetonitrile. Thus, allicin is present in AGE along with other anti-cepacia compounds.

4.7.5 Electron Microscopy

Scanning and transmission electron microscopy of *B. cepacia* revealed distortion of the bacterial cell structure after incubation with AGE. These morphological changes were observed after two hours incubation with AGE. The distortion of the cell membrane is similar to that observed by scanning electron microscopy of *C. albicans* treated with AGE (Ghannoum, 1988). In this study, cell leakage studies confirmed that the integrity of the yeast cell envelope was affected. AGE decreased the total lipid concentration and altered the fatty acid composition of *C. albicans*. Damage to the lipids in the yeast cell envelope would explain the distortion observed. Another study showed that AGE causes limited inhibition of protein and nucleic acid synthesis in *C. albicans*, whereas lipid synthesis ceased (Adetumbi et al, 1986). The outer membrane of *B. cepacia* is made up of similar fatty acids to those found in the membrane of *C. albicans*. *B. cepacia* produces an unusual range of polar lipids which include two forms of both phosphatidylethanolamine and ornithine amide lipid, along with phosphatidylglycerol, bis(phosphatidyl)glycerol and an uncharacterised glycolipid (Yabuuchi et al, 1992; Taylor et al, 1998). The most common fatty acids present in strains of *B. cepacia* are palmitic acid (16:0), oleic acid (18:1) and C17 and C19 cyclopropane acids (Taylor et al, 1998; Cox and Wilkinson, 1989).

If concentrations of palmitic and oleic acids are increased in *B. cepacia* when incubated in the presence of AGE, as is observed in *C. albicans*, the change in composition of fatty acids and lipids present within the outer membrane could lead to disruption of the outer membrane. Thus, at low concentrations garlic may inhibit

protein synthesis of bacteria, but at higher concentration it may also interfere with the production of fatty acids and lipids leading to the disruption of the outer membrane. This may be the irreparable lesion eluded to previously in this thesis. Resistance of *B. cepacia* to many conventional antibiotics is mediated by decreased outer membrane permeability (Burns and Clark, 1992). Small porin channels in the outer membrane of *B. cepacia* retard the diffusion of hydrophilic antibiotics to their targets making the organism resistant to β -lactam antibiotics (Parr et al, 1987; Arnoff, 1988) and chloramphenicol (Burns et al, 1989).

The unusual profile of polar lipids and fatty acids in *B. cepacia* may also confer unusual properties on the cell envelope thus contributing to antibiotic resistance (Cox and Wilkinson, 1989). Compounds present in AGE may have difficulty crossing the outer membrane of *B. cepacia*. Thus, it could be speculated that the outer membrane needs to be compromised in some way before inhibitory concentrations of the antibacterial compounds present within AGE can enter the cell.

AGE could exert its bactericidal action against *B. cepacia* in one of two ways. By some as yet unknown mechanism, AGE could damage the lipids of the cell membrane without entering the cell. After the membrane is weakened, thiosulphates could enter the cell more easily, causing further damage to cellular lipids and disruption of RNA synthesis. Alternatively, small quantities of thiosulphates may be able to enter *B. cepacia* through the pores present within the cell membrane and attack the sulphhydryl enzymes responsible for lipid synthesis, thus causing disruption of the cell membrane. As before, once the cell membrane is disrupted, larger quantities of AGE would be able to enter the cell causing greater

interference with lipid and RNA synthesis and also to any other cellular reaction catalysed by sulfhydryl enzymes, ultimately leading to cell death.

Garlic extracts have been shown to have a beneficial effect upon lipids present in the bloodstream of man. There may be similarities between the inhibition of lipids present within micro-organisms and the inhibition of lipids present within the human bloodstream. Ingestion of garlic has been shown to decrease levels of lipoproteins which circulate in the blood in the form of low density lipoprotein (LDL) and cholesterol. The formation of anti-atherogenic high density lipoprotein (HDL) is increased at the expense of LDL. Allicin is the compound thought to be responsible for this therapeutic effect as garlic preparations which do not contain allicin have been shown to be ineffective at lowering LDL and cholesterol concentrations (Reuter et al, 1995). Allicin may also lower cholesterol concentrations by the inhibition of cholesterol biosynthesis. At low concentrations allicin and ajoene have been shown to inhibit sterol biosynthesis in human HepG2 cells at the level of β -hydroxy- β -methyl-glutaryl-coA reductase and to inhibit the later stages of cholesterol biosynthesis at higher concentrations (Reuter et al, 1995). Both allicin and ajoene are converted to allyl-mercaptan immediately in blood, thus never reach the liver to affect cholesterol biosynthesis. However, allyl-mercaptan is capable of enhancing palmitate-induced inhibition of cholesterol synthesis (Gebhart, 1995).

It appears that allicin or allyl-mercaptan affects lipid production in both mammalian and yeast cells and may also affect the lipids present in the outer membrane of cells of *B. cepacia*. Lipid production may be affected by inhibition of sulfhydryl enzymes crucial for lipid synthesis or by some as yet unknown mechanism. The mechanism of inhibition could be shared in both micro-organisms and humans.

Further investigation would be necessary to ascertain if inhibition of production or damage to the lipids of the outer membrane of *B. cepacia* is causing the distortion seen in the electron microscopy photographs, and ultimately the destruction of cell structure.

CHAPTER 5 OIL EXTRACTS OF PLANTS

5.1 Screening of 21 plant essential oils for antimicrobial activity.

In a preliminary study, two strains of *B. cepacia* from different genomovars, were tested for susceptibility to 21 essential oils. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each isolate was determined for an inoculum of approximately 1×10^5 cfu/ml. A control for each isolate was prepared containing no essential oil. The control cultures grew to concentrations of approximately 1×10^9 cfu/ml over an 18 hour incubation period.

Table 5.1 Activity of 21 plant oils against two strains of *B. cepacia*

	C1052		J2315	
	MIC %	MBC %	MIC %	MBC%
Aniseed	1	>1	0.5	>1
Bay	0.05	0.1	0.05	0.1
Blackberry	>1	>1	>1	>1
Cinnamon	0.05	0.1	0.1	0.1
Clove	1	1	1	1
Cranberry	>1	>1	>1	>1
Eucalyptus	0.25	0.25	0.5	>1
Fennel	0.1	0.1	0.05	0.05
Garlic	0.25	0.5	0.1	0.5
Lemon	>1	>1	>1	>1
Lime	>1	>1	>1	>1
Mint	1	1	1	>1
Onion	>1	>1	1	>1
Orange	>1	>1	>1	>1
Peppermint	>1	>1	>1	>1
Rosemary	0.25	0.5	0.5	1
Raspberry	>1	>1	>1	>1
Spearmint	1	1	1	1
Strawberry	>1	>1	>1	>1
Tea Tree	0.25	0.25	0.25	0.25
Thyme	0.05	0.05	0.05	0.05

Eight of the oils tested showed no activity against the two strains of *B. cepacia* at concentrations of 1% or less. Thirteen oils had some activity against the strains and the six most active oils, bay, cinnamon, fennel, garlic, tea tree and thyme were selected for further study.

5.2 Susceptibility testing

Nineteen strains of the *B. cepacia* panel were tested for susceptibility to the six most active plant essential oils chosen from the screening studies. Approximately 1×10^5 cfu/ml of each bacterial strain, contained in nutrient broth were challenged with concentrations of plant oil between 0.01% and 1%. The suspensions were mixed thoroughly for 30 seconds before incubation in an orbital incubator to ensure dispersion of the oil throughout the broth. The MICs and MBCs are listed in tables 5.2 and 5.3 below. Cultures of *P. aeruginosa* (NCTC 10662), *E. coli* (J2408) and *S. aureus* (J2407) were included in the study as controls.

Table 5.2 MIC (%) of six plant essential oils against strains of *B. cepacia* and controls

	Thyme	Cinnamon	Bay	Fennel	Garlic	Tea Tree
A507	0.01	0.10	0.15	0.025	0.25	0.25
C1052	0.01	0.10	0.05	0.05	0.25	0.25
C1511	0.01	0.10	0.10	0.05	0.10	0.25
C1576	0.01	0.05	0.15	0.15	0.05	0.25
C1632	0.01	0.05	0.05	0.01		
C1773	0.01	0.05	0.05	0.05	0.05	0.10
C1962	0.01	0.05	0.05	0.10	0.25	0.25
C1963	0.01	0.05	0.05	0.05	0.05	0.10
C1964	0.01	0.05	0.05	0.05	0.05	0.10
J415	0.01	0.10	0.05	0.10	0.25	0.25
J419	0.01	0.05	0.05	0.05	0.10	0.10
J673	0.01	0.05	0.10	0.05	0.10	0.10
J2315	0.01	0.15	0.05	0.10	0.10	0.10
J2421	0.01	0.05	0.05	0.05	1.00	0.25
J2502	0.01	0.05	0.10	0.05	0.10	0.10
J2503	0.01	0.05	0.05	0.05	0.25	0.10
J2524	0.01	0.05	0.05	0.05	0.25	0.10
J2537	0.01	0.15	0.15	0.025	0.50	0.25
J2552	0.01	0.05	0.05	0.05	0.05	0.10
CONTROLS						
J2407	0.05	0.05	0.05	0.10	0.25	0.25
J2408	0.05	0.05	0.10	0.10	1.00	0.05
NCTC10662	1.00	1.00	0.50	>1.00	1.00	1.00

Table 5.3 MBC (%) of six plant essential oils against strains of *B. cepacia* and controls

	Thyme	Cinnamon	Bay	Fennel	Garlic	Tea Tree
A507	0.025	0.200	0.200	0.050	0.500	0.250
C1052	0.010	0.100	0.050	0.100	0.500	0.250
C1511	0.025	0.250	0.150	0.150	0.250	0.250
C1576	0.010	0.150	0.250	0.150	0.500	0.250
C1632	0.010	0.050	0.050	0.010		
C1773	0.010	0.050	0.050	0.050	0.100	0.100
C1962	0.025	0.150	0.150	0.100	>1.000	0.250
C1963	0.010	0.050	0.050	0.050	0.250	0.250
C1964	0.010	0.050	0.100	0.050	0.500	0.250
J415	0.025	0.100	0.150	0.250	0.500	0.250
J419	0.025	0.100	0.050	0.100	0.250	0.100
J673	0.010	0.050	0.100	0.100	0.500	0.250
J2315	0.010	0.250	0.250	0.100	0.500	0.250
J2421	0.010	0.050	0.050	0.050	>1.000	0.250
J2502	0.010	0.050	0.100	0.050	0.250	0.100
J2503	0.025	0.050	0.050	0.150	0.500	>1.000
J2524	0.010	0.050	0.050	0.100	0.250	0.100
J2537	0.025	0.500	0.150	0.025	1.000	0.250
J2552	0.010	0.050	0.050	0.100	0.250	0.250
CONTROLS						
J2407	0.500	0.100	0.050	>1.000	1.000	0.250
J2408	0.050	0.050	0.100	0.100	1.000	0.050
NCTC10662	1.000	>1.000	1.000	>1.000	1.000	1.000

A summary of the results is displayed in Table 5.4. Thyme oil was found to be the most antimicrobial oil. Fennel, cinnamon and bay oils have similar activity against the strains tested. Garlic and tea tree oils are moderately less active than the other oils tested. In general, none of the control organisms tested were as susceptible as the *B. cepacia* strains to the range of oils. *S. aureus* (J2407), however, was quite susceptible to bay oil and *E. coli* (J2408) appeared relatively sensitive to thyme and cinnamon oils. *P. aeruginosa* (NCTC 10662) was the most resistant of the strains tested. A 1% concentration of thyme, bay, garlic or tea tree oils was required to inhibit the growth of this strain.

Table 5.4 Summary of susceptibility testing for *B. cepacia* strains

	MIC 90	MIC range	MBC range
Thyme	0.01%	All 0.01%	0.01% - 0.025%
Fennel	0.1%	0.01% - 0.15%	0.01% - 0.15%
Cinnamon	0.1%	0.05% - 0.15%	0.05% - 0.5%
Bay	0.15%	0.05% - 0.15%	0.05% - 0.25%
Tea tree	0.25%	0.1% - 0.25%	0.1% - >1%
Garlic	0.25%	0.05% - 1%	0.1% - >1%

5.3 HPLC analysis of thyme oil

HPLC was used to confirm that thymol was present in the thyme essential oil used in this thesis. Thymol absorbs light at a wavelength of 275nm, therefore this wavelength was chosen to separate thyme oil by HPLC. Thyme oil and thymol were analysed on a continuous linear gradient of acetonitrile in 0.1% (vol/vol) trifluoroacetic acid designed to give a clear separation of the compounds present. Separation of thyme oil yielded four peaks. The largest of which, peak 3

corresponds to the peak produced when thymol is analysed under identical conditions. This suggested that peak three was the thymol fraction of thyme oil.

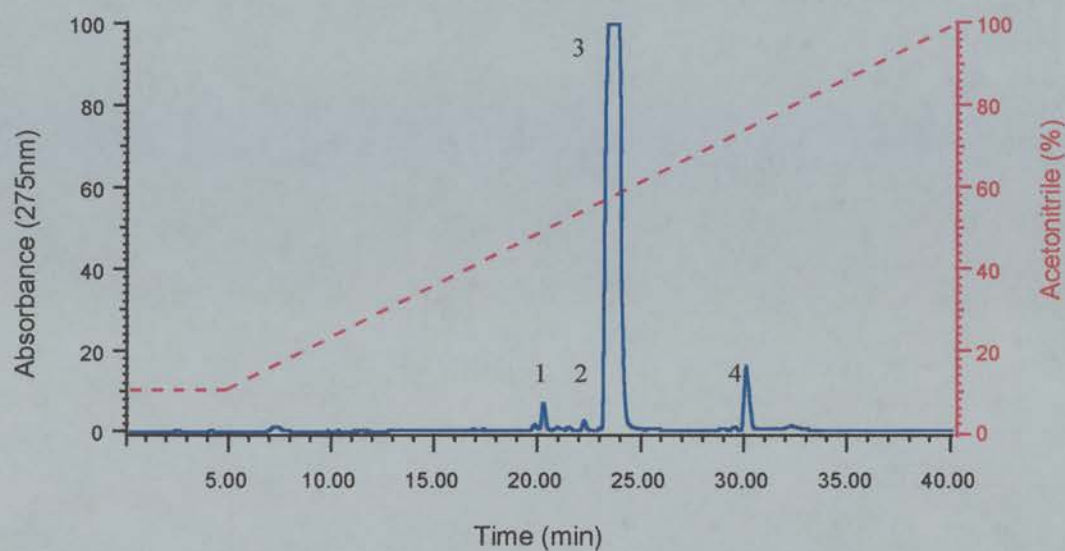


Fig 5.1 Separation of thyme oil by HPLC at 275nm

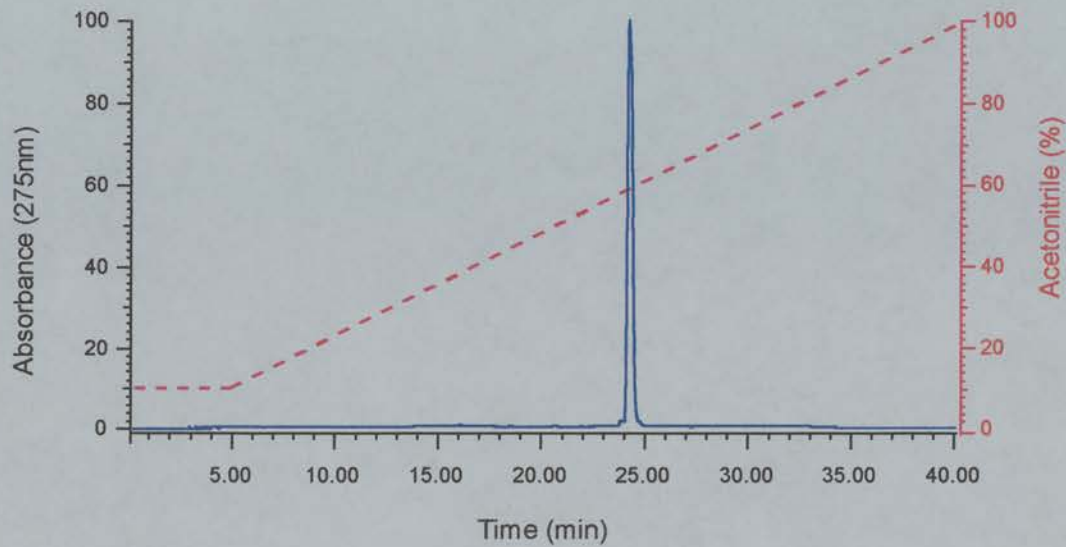


Fig 5.2 Separation of thymol by HPLC at 275nm

It can be seen from figures 5.1 and 5.2 above that thymol is likely to present in the thyme essential oil studied in this thesis. Carvacrol and thymol analysed at the same absorbance and under identical condition produced almost identical HPLC traces. Thus, either compound, or more likely a combination of both compounds seems to be present in the thyme oil analysed in this thesis. Results are shown in the appendix.

5.4 Susceptibility of *B. cepacia* to thymol

Since thymol is a major constituent of thyme oil, the MIC and MBC of a pure preparation of thymol were determined for the panel of 20 *B. cepacia* strains.

Thymol does not dissolve well in water alone therefore 5% ethanol was used to aid dispersion. A concentration of ethanol greater than the maximum amount present in any sample was tested for antimicrobial activity. Thus it was found that 5% ethanol alone did not have an antibacterial effect. Each strain of *B. cepacia* was tested for susceptibility to concentrations of thymol between 100 and 500µg/ml.

Table 5.5 Susceptibility of strains of *B. cepacia* to thymol (µg/ml)

	MIC	MBC		MIC	MBC
C1052	200	200	J673	300	300
C1511	300	400	J2315	200	300
C1576	300	300	J2421	200	300
C1632	300	300	J2502	200	200
C1773	200	300	J2503	300	300
C1962	200	300	J2524	300	300
C1963	200	200	J2537	300	300
C1964	200	300	J2552	300	300
J415	300	300	J2742	300	300
J419	200	300	A507	200	200

Thymol at a concentration of 500µg/ml did not inhibit the growth of *P. aeruginosa* NCTC 10662. Concentrations of between 200 and 400µg/ml were sufficient to inhibit the growth of the *B. cepacia* strains tested.

5.5 Killing curves

The declines of both logarithmic and stationary phase populations of *B. cepacia* challenged with thyme oil or thymol were studied. The period required for the minimum bactericidal concentration of each substance to eradicate 10^5 cfu/ml of cultures of a clinical or environmental strain of *B. cepacia*, either in stationary or logarithmic phase of growth, is shown below. Each experiment was carried out three times and on each occasion sampling was carried out in triplicate. The standard error of the mean was calculated and error bars are shown. Figures 5.3 and 5.4 show the activity of thyme oil against stationary phase cultures of the two *B. cepacia* strains.

Fig 5.3 Time related killing of stationary phase cultures of *B. cepacia* J2315 in the presence of thyme oil.

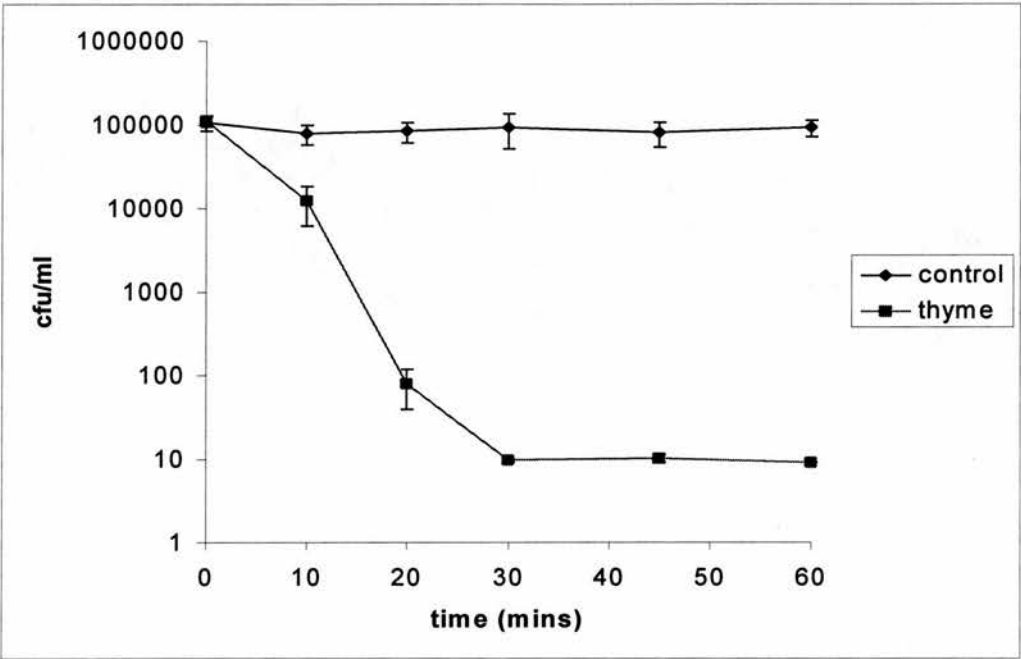


Fig 5.4 Time related killing of stationary phase cultures of *B. cepacia* J2421 in the presence of thyme oil.

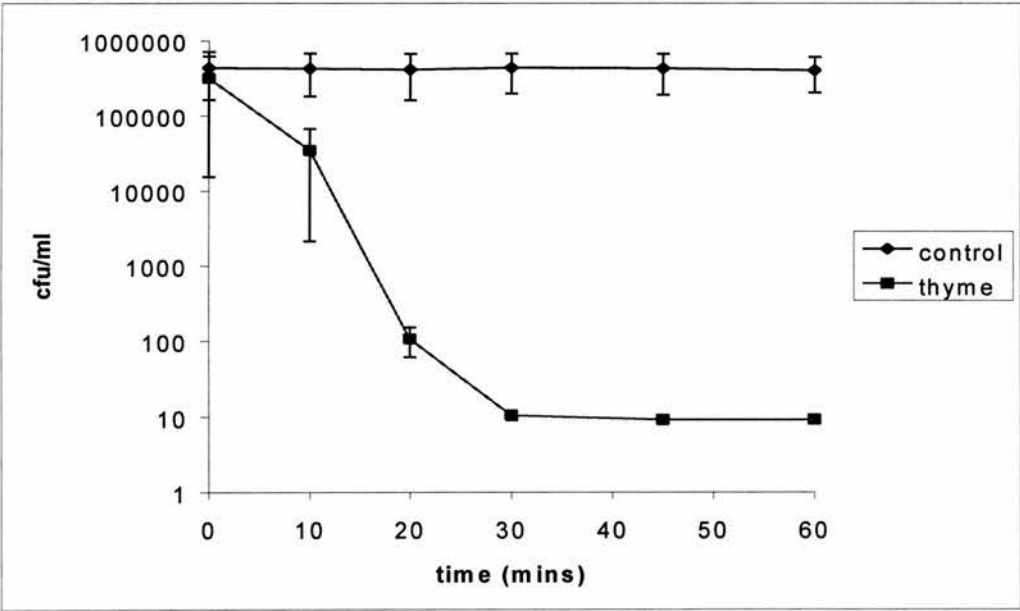


Fig 5.5 Time related killing of log phase cultures of *B. cepacia* J2315 in the presence of thyme oil.

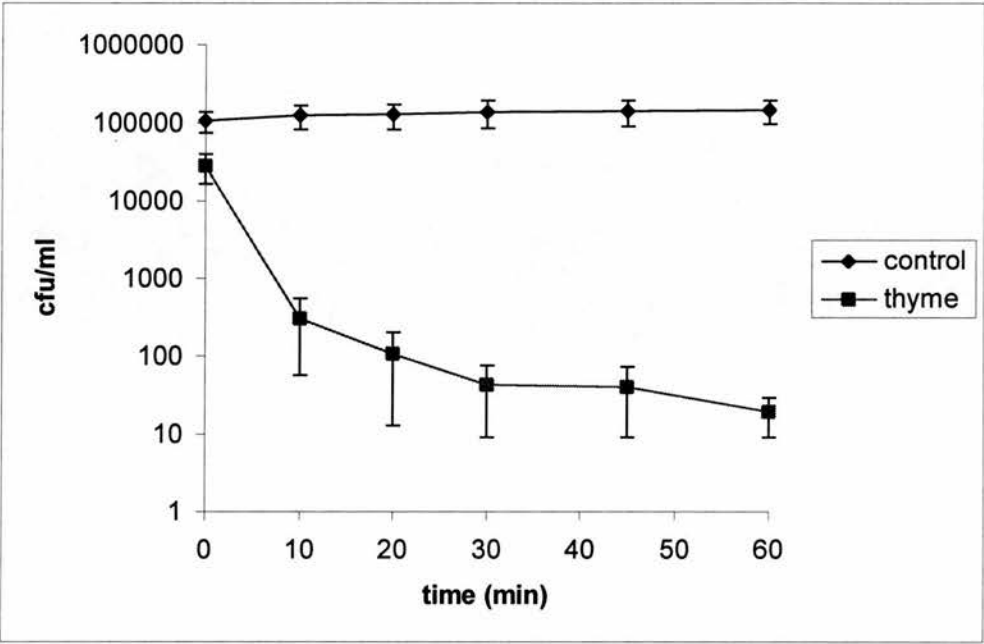
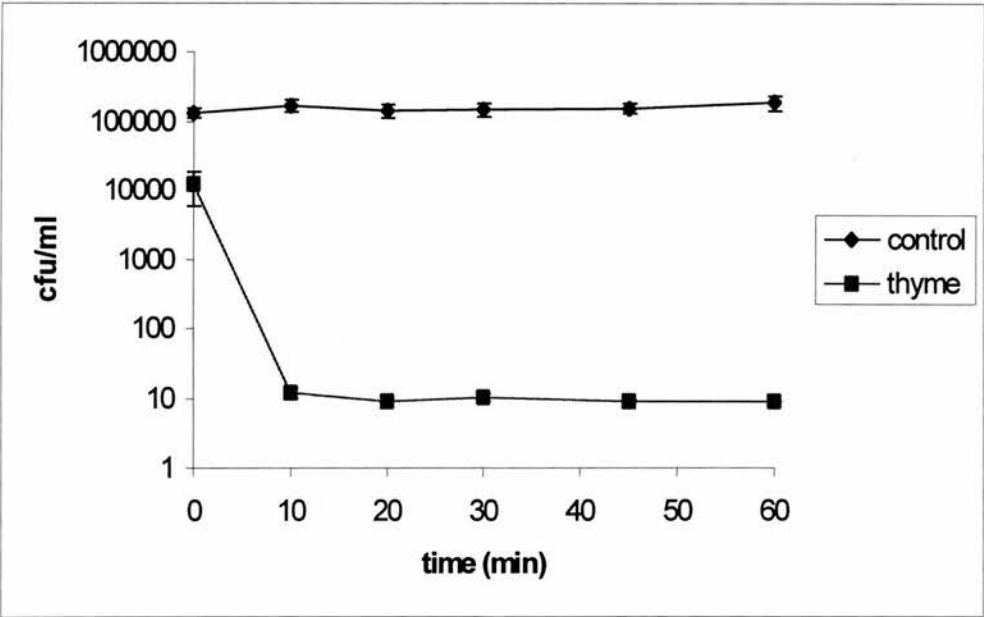


Fig 5.6 Time related killing of log phase cultures of *B. cepacia*.J2421 in the presence of thyme oil.



Figures 5.5 and 5.6 show the activity of thyme oil on log phase cultures of two strains of *B. cepacia*. Compared with the killing curves observed for stationary phase cultures, it can be seen that log phase cultures are killed slightly more swiftly. Approximately twenty minutes contact with oil was necessary to eradicate stationary phase cultures whereas log phase cultures were 'sterilised' in ten minutes.

At MBC (300µg/ml), thymol did not kill the two strains of *B. cepacia* tested to the same extent as thyme oil. At 300µg/ml however, thymol decreased bacterial cfus within 90 minutes by 40% (data not shown). In order to mimic the rate of antimicrobial activity of thyme oil against *B. cepacia*, 1000µg/ml of thymol was required for J2315 and 800µg/ml for J2421. The results of these experiments are shown in figures 5.7, 5.8, 5.9 and 5.10. Once again, the experiments were carried out on three occasions and on each occasion sampling was carried out in triplicate.

Fig 5.7 Time related killing of stationary phase cultures of *B. cepacia* J2315 in the presence of thymol.

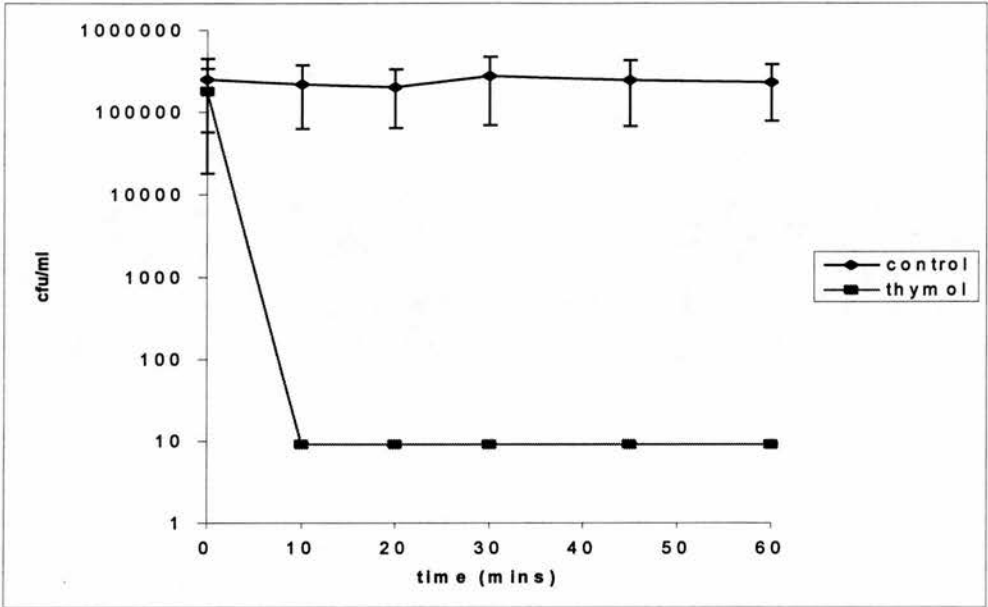


Fig 5.8 Time related killing of stationary phase cultures of *B. cepacia* J2421 in the presence of thymol.

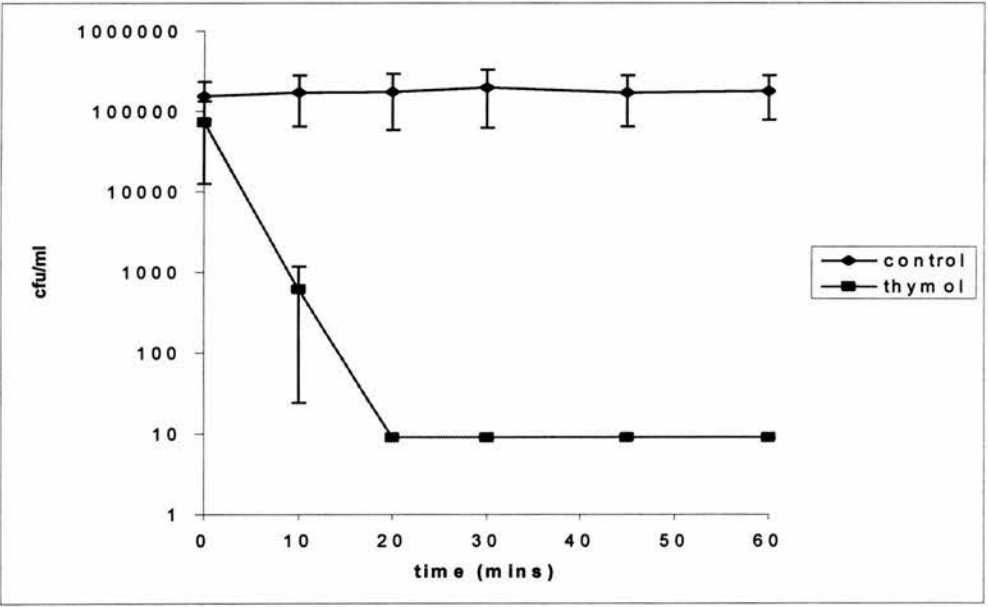


Fig 5.9 Time related killing of log phase cultures of *B. cepacia* J2315 in the presence of thymol.

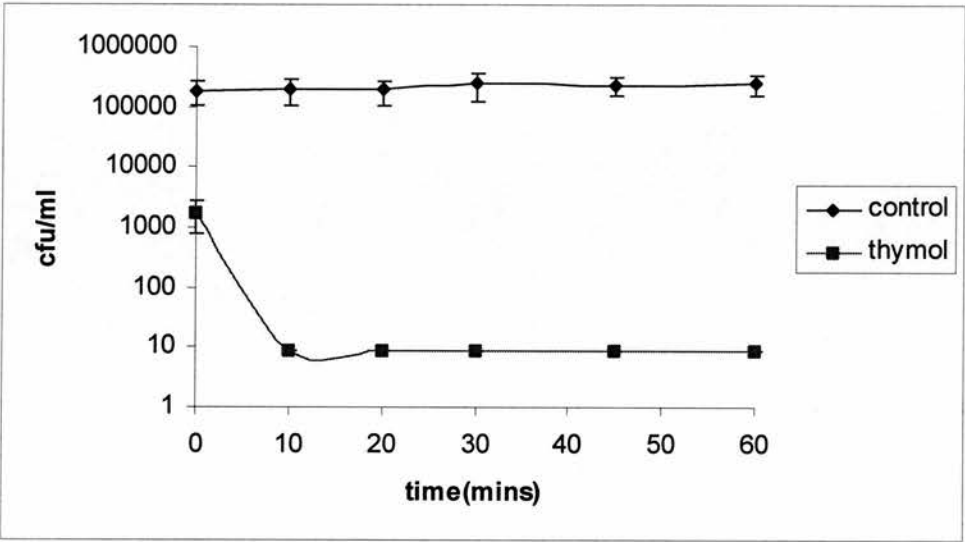
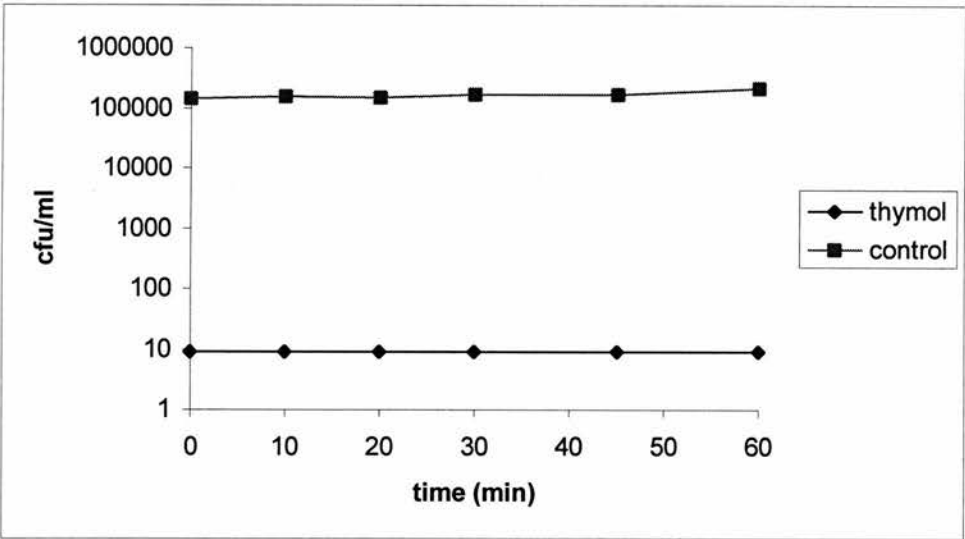


Fig 5.10 Time related killing of log phase cultures of *B. cepacia* J2421 in the presence of thymol.



5.6 Killing curves in the absence of a carbon source

To determine if cells must be actively growing for thyme oil to have a bactericidal effect, killing curves were carried out in PBS instead of nutrient broth *i.e.* in the absence of a carbon source.

Figures 5.11 and 5.12 show that there was no noticeable difference in the decline of these strains upon challenge with thyme oil in PBS or nutrient broth. Therefore, it can be assumed that the cells do not need to be actively growing for thyme oil to produce a bactericidal effect.

Figure 5.11 Time related killing in the absence of a carbon source of a log phase culture of *B. cepacia* J2315 in the presence of thyme oil.

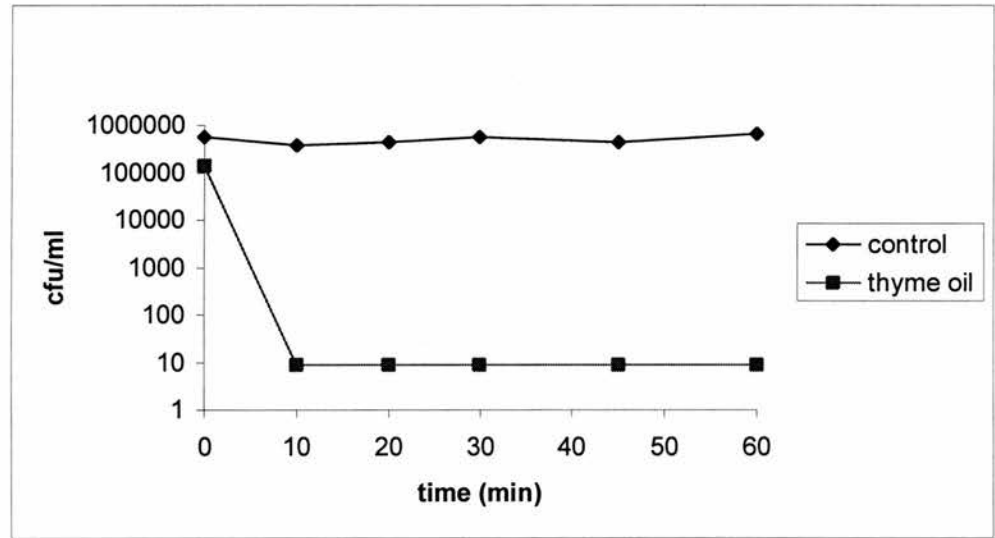
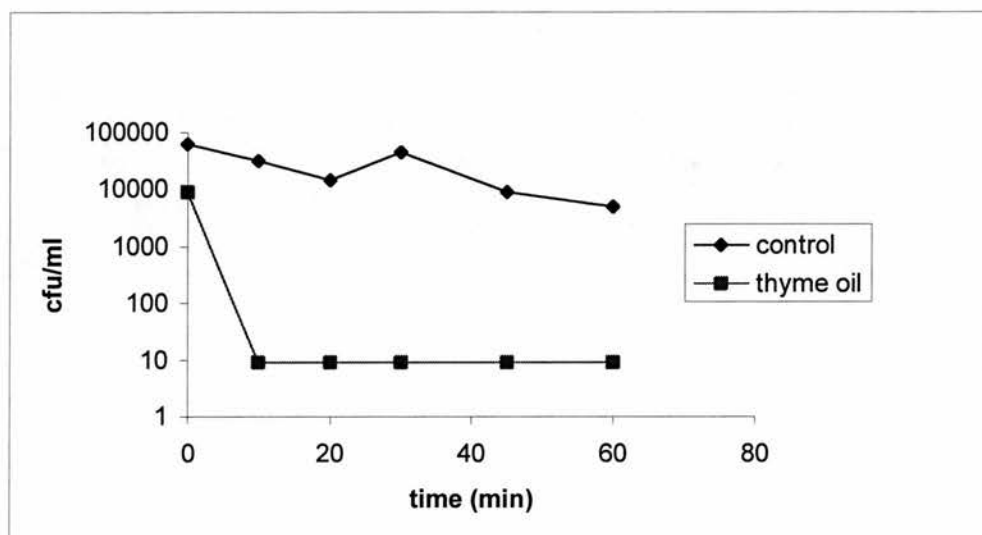


Fig 5.12 Time related killing in the absence of a carbon source of a log phase culture of *B. cepacia* J2421 in the presence of thyme oil.



5.7 Dose response curves

The bactericidal activity of thyme oil and thymol were measured at a range of concentrations for two strains of *B. cepacia* in nutrient broth. Bacterial numbers were shown to decrease steadily with increasing concentrations of thyme oil or thymol until all detectable cells were eradicated.

Fig 5.13 Survival of *B. cepacia* J2315 (initial concentration 4.4×10^5 cfu/ml) and J2421 (initial concentration 7.52×10^6 cfu/ml) after exposure to thyme oil for 5h and 4h respectively in iso-sensitest broth at 37°C.

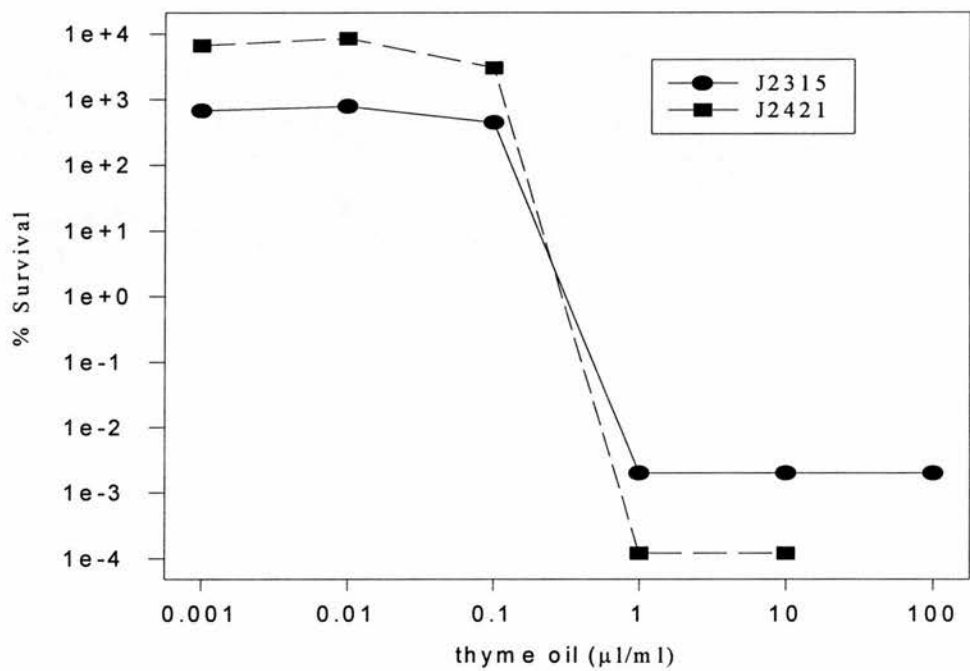
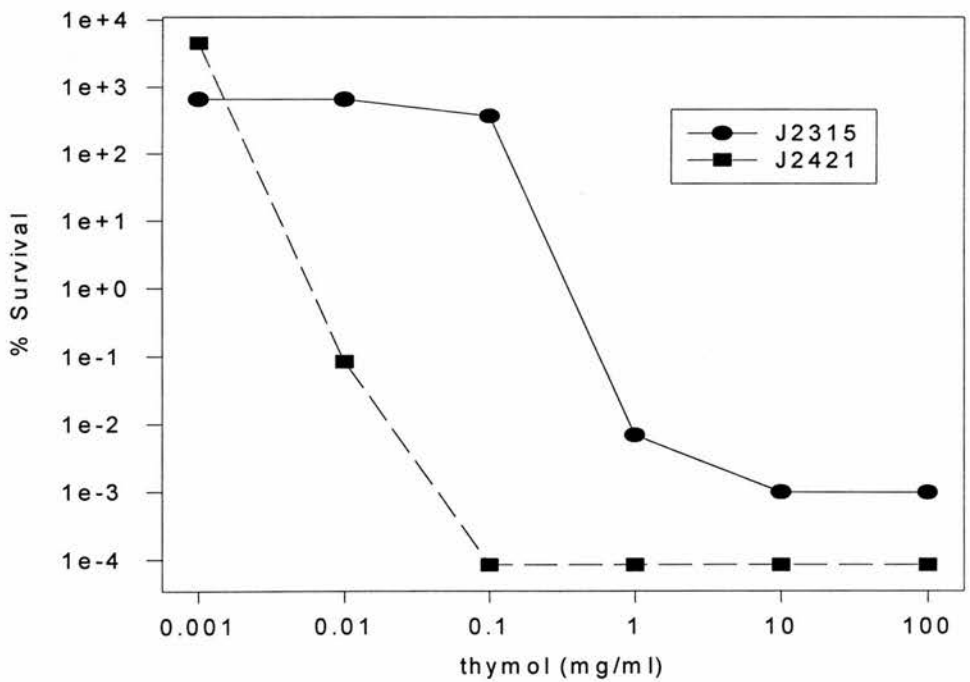


Fig 5.14 Survival of *B. cepacia* J2315 (initial concentration 7.32×10^5 cfu/ml) and J2421 (initial concentration 1.06×10^5 cfu/ml) after exposure to thymol for 5h and 4h respectively in iso-sensitest broth at 37°C.



5.8 Electron Microscopy

Transmission electron microscopy

Transmission electron microscopy revealed significant changes after only three minutes incubation of cells of strain J2315 with thyme oil. No intact bacteria could be observed after this short incubation period.

Scanning electron microscopy

Scanning electron microscopy revealed only limited numbers of cells of strain J2315 survived two minutes incubation with thyme oil. Figure 5.16 and figure 5.17 both show damaged bacterial cells, some with blebs of material on their surface. Cells of J2315 incubated in thyme oil also appear shorter than the control cells of this strain shown in figure 5.15.

Fig. 5.15 Control cells of *B. cepacia* J2315.



Fig. 5.16 *B. cepacia* J2315 after two minutes incubation with thyme oil



Fig 5.17 *B. cepacia* J2315 after two minutes incubation with thyme oil



5.9 Discussion: Oil extracts of plants

5.9.1 Screen of 21 essential oils

Of the essential oils tested in this study, only 50% showed inhibitory activity against *B. cepacia* at concentrations of 1% or less. None of the seven fruit essential oils, nor onion oil, showed any activity. Peppermint oil also had no anti *cepacia* activity at the concentrations tested, however oils of mint and spearmint were inhibitory at concentrations of 1%. The other ten oils tested did show inhibitory activity against *B. cepacia*. These oils were mainly isolated from herbs, and many contain the same or similar chemicals. Bay, cinnamon and clove oils contain mainly the phenol eugenol. Fennel and aniseed oils contain mostly trans-anethole. Additional compounds, present within these oils may contribute to their antimicrobial activity as bay, cinnamon and fennel oils are much more inhibitory to the growth of the *B. cepacia* strains tested than oils from clove and aniseed. However, a greater number of strains need to be tested before firm conclusions are drawn. Rosemary and eucalyptus oils comprise mainly 1,8-cineole, a cyclic ether, and both oils showed a similar level of antibacterial activity against the two *B. cepacia* strains tested. Garlic, tea tree and thyme oils are composed of compounds unique amongst the oils tested. Garlic oil is composed of a number of sulphur containing compounds and tea tree oil contains mainly terpinen-4-ol, a terpene alcohol. Thyme oil, the most active of all the oils tested against *B. cepacia* contains the phenols, thymol and carvacrol. Oils of bay, cinnamon, fennel, garlic, tea-tree and thyme were selected for further investigation as they exhibited the greatest activity against the strains of *B. cepacia* tested in the preliminary screen.

5.9.2 Activity of selected oils against the *B. cepacia* panel

Thyme oil was the most inhibitory of the oils tested against *B. cepacia* with the batch tested having a MIC of 0.01% for all strains tested. The MBCs ranged from 0.01% to 0.025%. Many studies of the antibacterial and antifungal activity of thyme oil and its main active constituents thymol and carvacrol, have been reported previously. Thyme oil has powerful antibacterial and antifungal activity against a wide range of organisms. In a study of fifty plant essential oils against single strains of 25 genera of bacteria, Deans and Ritchie (1987) found thyme oil to be active against 23 of the genera tested. Only two genera, represented by *Clostridium sporogenes* and *Leuconostoc cremoris*, were resistant to thyme oil. Only angelic and bay oils were found to inhibit more bacteria than thyme oil. It is important to note that this study was carried out using agar diffusion, a technique which does not provide as accurate results due the difficulties in obtaining uniform distribution because of the hydrophobicity of essential oils (Janssen et al, 1987). In a more directly comparable study carried out using methods similar to those used to test conventional antibiotics, thyme oil was again shown to be highly active, inhibiting eight out of the ten organisms tested (Hammer et al,1999). Only *P. aeruginosa* and *S. typhimurium* were resistant to up to 2% v/v concentrations of thyme oil. Thyme oil has also been shown to have activity against organisms highly resistant to conventional antibiotics, such as MRSA and vancomycin resistant enterococci using a broth dilution method (Nelson, 1997). Although most other studies of the antibacterial activity of thyme oil have unfortunately been carried out using agar or disk diffusion methods, they provide comparative and qualitative data for the activity of thyme oil against a wide

variety of organisms. For example, thyme oil was found to be the most active of six essential oils tested against Gram-positive, Gram-negative, acid fast bacteria and yeast at concentrations of 1.25mg/ml and less (Farag, 1989). Thyme oil was also the most active of the oils tested in a study of inhibition of oral bacteria (Meeker, 1988). Thyme oil, along with bay and cinnamon oils, was again the most active of 23 oils tested against food-borne pathogens (Smith-Palmer et al, 1998). Other studies have concentrated on the considerable activity of thyme oil against fungi and yeasts (Conner and Beauchat, 1984; Llewellyn et al, 1981). Dried, alcoholic and aqueous extracts of thyme have also been shown to possess antimicrobial activity (Beuchat, 1976; Hitokoto et al, 1980; Huhtanen, 1980; Tabak et al, 1996).

Some investigators have reported that thymol alone is active against *S. typhimurium*, *S. aureus*, *V. parahaemolyticus* (Karpainar and Aktung, 1987), some oral bacteria (Shapiro et al, 1994) and fungi (Thompson, 1990; Buchannan and Shepherd, 1981).

Carvacrol, the other main antibacterial compound present in thyme oil has been shown to have activity against *B. cereus*. It should be noted that studies of the individual components of essential oils are not directly comparable with studies of whole oil as interactions between the compounds within the oil may have additive, synergistic or antagonistic contributions to the antimicrobial effect.

Two of the other oils tested in this thesis also contain high levels of phenols. These are cinnamon leaf and bay oils which are both mainly composed of eugenol. These oils were found to be highly active against the *B. cepacia* panel with a similar range of MICs and MBCs. Indeed, the susceptibilities of approximately 60% of the strains to cinnamon and bay oils were identical. Where differences did exist it, it is possible that other compounds present within these oils, perhaps with different modes of

action, may contribute or detract from the antibacterial activity against individual strains. It is unlikely that the differences are due to a particular oil containing a higher concentration of eugenol as no oil was consistently more active than the other. Although this thesis focused on *B. cepacia*, in general the antibacterial activities of bay and cinnamon oils observed were consistent with other studies. However, cinnamon essential oil can be produced from either the leaves or the bark of this plant and in most studies, the type of cinnamon oil used is not specified (Aureli et al, 1992; Deans and Ritchie, 1987; Maruzzella and Henry, 1958; Yousef and Twail, 1980; Llewellyn et al, 1981). In this thesis, activity of oil of cinnamon leaf was investigated. Cinnamon bark essential oil contains between 55 and 75% cinnamaldehyde, which is not present in cinnamon leaf oil. It also contains much less eugenol than cinnamon leaf oil (Tissarand and Balacs, 1995). Smith-Palmer and co-workers (1998) have shown bay and cinnamon leaf oils to be active against *E. coli*, *S. aureus*, *L. monocytogenes* and *S. enteritidis* at concentrations of 1% or less. All species tested with the exception of *E. coli* were susceptible to different concentrations of bay and cinnamon oils. Bay oil was found to be active against *Acinetobacter baumannii*, *Aeromonas sobria*, *C. albicans*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhimurium*, *S. marcescens* and *S. aureus* at concentrations of 1% or less (Hammer et al, 1999). This confirmed the antibacterial activity of bay oil, previously observed by Nadal et al (1973) and other investigators, who used agar diffusion techniques. Eugenol was found to inhibit the growth of *Staphylococcus*, *Micrococcus*, *Bacillus* and *Enterobacter* isolates at a concentration of 750µg/ml (Moleyar and Narasimham, 1992). In a separate study, 100µg/ml eugenol was required to completely inhibit *S. typhimurium*, *S. aureus* and

V. parahaemolyticus (Karapinar and Aktug, 1987). A mixture of cinnamaldehyde and eugenol was found to be more active than the same concentrations of these compounds alone against *Staphylococcus*, *Micrococcus*, *Bacillus* and *Enterobacter* isolates (Moleyar and Narasimham, 1992).

In this thesis, sweet fennel oil proved to be marginally more active than cinnamon and bay oils against the *B. cepacia* panel. This oil is composed mainly of trans-anethole, Anethole has previously been reported to be antimicrobial against strains of *S. aureus*, *S. typhimurium* and *V. parahaemolyticus* at concentrations of 500µg/ml (Karapinar and Aktug, 1987). However, Smith -Palmer et al (1998) found fennel oil to have no activity against a selection of food poisoning organisms at concentrations of less than 1%. Other studies have shown fennel to be antimicrobial against a limited range of bacteria and fungi (Deans and Ritchie, 1987; Yousef and Twail, 1980; Conner and Beauchat, 1984). Fennel oil is surprisingly active against *B. cepacia* considering its limited range of activity against other bacteria and fungi, many of which are considerably more sensitive to conventional antimicrobial agents. All the studies mentioned above were carried out in agar as opposed to broth which may account for the poor inhibitory activity observed.

Garlic and tea tree oils were less active against the *B. cepacia* panel strains than the other oils tested. Garlic oil is reported to contain mostly diallyl disulphide and other sulphur containing compounds, discussed extensively in chapter 4. Garlic oil is unlikely to contain allicin, as this volatile compound would not survive prolonged storage at room temperature. Therefore, other compounds present within the oil may be responsible for anti-*cepacia* activity. Tea tree oil also has activity against the strains of *B. cepacia* studied. Although it was not as active as some of the other oils

tested, the range of activity of tea tree oil was similar to that observed in previous studies against MRSA (Nelson, 1997). Lower concentrations of tea tree oil were required to inhibit certain oral bacteria, considered more sensitive to conventional antibiotics (Shapiro et al, 1994).

From the results obtained in this thesis, it can be assumed that eugenol is unlikely to be as active against *B. cepacia* strains as a combination of thymol and carvacrol and may not even be as active as either of these compounds alone. Compounds present within fennel oil, most probably tran-anethole, also have considerable anti-*cepacia* activity. Of all the oils, only garlic and tea tree were unable to completely inhibit all the *B. cepacia* strains investigated at concentrations of 1% or less.

Some *B. cepacia* strains were susceptible to relatively low concentrations of some oils; those included strains C1773, C1963/C1964, J419, J673, J2502 and J2552. In contrast, strains A507, C1511, C1576, J2315 and J2537 required higher concentrations to inhibit growth. Susceptibility or resistance was not associated with any particular *B. cepacia* genomovar or with the source of the isolate. However, it should be noted that not all genomovars were represented in this study. In contrast, strains which were least sensitive to the oils were those exhibiting most resistance to conventional antibiotics. Those included strains C1576 and J3215 which were resistant to all the antibiotics tested and C1511 and J2537 which were sensitive to only two conventional antibiotics.

Strains of *B. cepacia* which were most sensitive to the plant oils were also sensitive to many of the conventional antibiotics studied. C1773, J419, C1963 and C1964 are clinical *B. cepacia* isolates sensitive to two or three of the conventional antibiotics. Two of the strains (J673 and J2502) were sensitive to four conventional antibiotics

and strain J2552, which exhibited the most sensitivity to conventional antibiotics, was also relatively sensitive to the plant oils.

Interestingly, strains of *B. cepacia* which were most sensitive to an AGE were not those which were most sensitive to garlic oil. Strains C1773, C1962, J2315, J2421 and J2742 were the most sensitive strains to AGE whereas C1576, C1773, C1963/C1964 and J2552 were the most sensitive to garlic oil. Only strain C1773 was particularly sensitive to both garlic extracts. Strain J2421 was especially sensitive to AGE, however this strain was the most resistant to garlic oil having a MIC of 1%. These results support the hypothesis that different compounds are responsible for the antibacterial activity of AGE and garlic oil.

Thyme oil was the most active of all the oils tested. The batch of thyme oil analysed, eradicated all cells of every strain at concentrations of 0.025% and was selected for further study.

5.9.3 HPLC analysis of thyme oil

Essential oils from a single plant can contain over 100 different chemical compounds. However, most individual oils contain one or two major compounds which shape the pharmacology of that particular oil (Tisserand and Balacs, 1995). HPLC was employed to investigate the batch of thyme oil for the presence of its major components thymol and carvacrol. Spectrophotometry revealed that thyme oil absorbed light mainly at 275nm, therefore this wavelength was used when the oil was examined by HPLC. Thyme oil, separated using a continuous linear gradient of acetonitrile, yielded four compounds. The largest peak corresponded to the peak produced when a thymol standard was analyzed under similar conditions. This

finding suggested that the large peak is the thymol fraction of thyme oil. Carvacrol produced an almost identical trace to thymol and thyme oil when analysed under identical conditions. This result was not surprising, as thymol and carvacrol are isomers. It would be impossible to separate these compounds by RP-HPLC and it may be that both thymol and carvacrol are contributing to the major peak observed when thyme oil is analysed under identical conditions.

When the material from the four thyme oil HPLC peaks was examined for activity against *B. cepacia*, only the thymol / carvacrol peak exhibited strong antimicrobial activity (data not shown).

5.9.4 Killing curves

Surprisingly, very few studies of the antimicrobial activity of plant essential oils or compounds present within essential oils have included time kill experiments. Studies by Kim and co-workers (Kim et al, 1995a; 1995b) did however, include experiments where the growth rate of *E. coli*, *E. coli* O157:H7, *Listeria monocytogenes*, *Vibrio vulnificus* and *Salmonella typhimurium* was measured in the presence and absence of eight plant essential oils components, including carvacrol and eugenol. In these experiments, absorbance at 540nm was used to measure bacterial growth. The use of 'turbidity' measurements to assess bacterial growth would include cells which were no longer viable. In addition, this technique is relatively insensitive for measuring small bacterial populations.

Similar experiments, measuring the growth of *B. cereus* in the presence of different concentrations of carvacrol, were carried out by Ultee et al (1998). In these studies, carvacrol at a concentration of 3mmol l⁻¹ was shown to cause no increase in

absorbance at 660nm *i.e.* no growth. Further experiments were carried out to ascertain the rate of killing of *B. cereus* by 2mmol l⁻¹ carvacrol. The concentration of *B. cereus* was reduced from 10⁷cfu/ml to 10⁴cfu/ml in only 20 minutes. This rate of killing is similar to that observed in this thesis when *B. cepacia* was exposed to thyme oil. Other studies, which have used serial dilutions to obtain counts of viable bacteria remaining after challenge by essential oil, have included the examination of the bactericidal activity of thyme oil against *Vibrio parahaemolyticus* (Beauchat, 1976) and *Listeria monocytogenes* (Aureli et al, 1992). The growth of *V. parahaemolyticus* was challenged with three essential oils, thyme, oregano and sassafras over a period of seven hours. Viable organisms were reduced from 10⁴ to almost zero by oregano oil within one hour, however recovery was observed, beginning after three hours and reaching almost 10² cfu/ml after seven hours. The effect of thyme oil was similar, yet not as pronounced. Bacterial numbers were reduced from 10⁴cfu/ml to 10²cfu/ml in one hour, after which growth recovered to the original level of 10⁴cfu/ml after seven hours. Sassafras oil was found to possess limited bactericidal activity. The rapid reduction of bacterial numbers observed by these two essential oils, both known to contain high levels of thymol and carvacrol, is consistent with other studies, and with the results observed in this thesis. In this thesis, thyme oil appeared to inhibit bacterial growth completely, however due to the limitations of the serial dilution technique it was not possible to determine bacterial numbers of less than 10¹/ml. Thus recovery may have been possible, although unlikely, if incubation had been prolonged. Aureli et al (1992) reported a slower rate of killing when five strains of *Listeria monocytogenes* were challenged with thyme, oregano, cinnamon, clove and pimento essential oils. Four hours incubation with

0.1% thyme and oregano essential oils was required for each of the five strains to be completely eradicated. Pimento and clove oils were found to have a faster rate of killing with some strains whereas the same concentration of cinnamon oil could only inhibit three of the five strains tested within four hours. The concentration of oil used by Aureli and co-workers was comparable with the minimum bactericidal concentration of essential oil for *B. cepacia* found in this thesis. However, longer incubation and slightly greater concentrations of thyme oil were required to eradicate *L. monocytogenes* than were required to eradicate *B. cepacia*. Thyme oil can vary dramatically in its composition between batches (Cruz et al, 1993), making reliable comparison difficult. In addition, the initial bacterial inocula used by Aureli et al were ten fold larger than those used in this thesis.

5.9.5 Mode of action

In this thesis, bacteria in the log phase of growth were killed more quickly than cells in stationary phase. Similar results have been noted for inhibition of *E. coli* by tea tree oil (Gustafson et al 1998; Cox et al 1998). However, the differences between inhibition of stationary and exponential phase cultures of *E. coli* were much more pronounced than with *B. cepacia*. In the case of *E. coli*, exponential phase cells were eradicated in 30 minutes, but 10^5 cfu/ml of stationary phase cells remained after 120 minutes. When bacteria, such as *Escherichia*, *Salmonella* and *Vibrio* reach stationary phase they become metabolically less active and more resistant to antimicrobials. Changes in membrane fatty acid composition and differences in the structure of the cell membrane synthesised during stationary phase may contribute to

the increased resistance of stationary phase cells to essential oils (Siegele and Kolter, 1992).

Killing of *B. cepacia* strains by thyme oil and thymol was swift, taking place within 30 minutes of addition of the antimicrobial. Further experiments carried out in PBS instead of ISB gave similar results with no detectable viable bacteria remaining after ten minutes. These results suggest that cells of *B. cepacia* do not need to be actively growing to be inhibited by thyme oil or thymol; thus, it is unlikely that these substances are inhibiting processes such as DNA, RNA or protein synthesis.

To produce the same rate of killing as thyme oil, concentrations of thymol of 1000µg/ml and 800µg/ml were required for J2315 and J2421 respectively. These concentrations appear high, as the MBC of thymol for both these strains was only 300µg/ml. It may be that other compounds present in thyme oil, such as carvacrol, a compound with known antimicrobial properties and an isomer of thymol, contribute to the rapid bactericidal effect.

The mechanism by which thymol and carvacrol exert their antimicrobial activity has been considered by other investigators. By studying compounds that inhibit the bactericidal action of thymol, Juven and co-workers formed a hypothesis for the mode of action of thymol based on the known mode of action of other phenols. They proposed that after crossing the bacterial cell wall, thymol interacts with periplasmic enzymes, and upon penetrating the cytoplasmic membrane interacts with membrane proteins by hydrophobic and hydrogen bonding, causing a back flow of protons across the cell membrane. This activity subsequently would affect cellular functions powered by the proton motive force (Juven et al, 1994). As a sudden decrease in bacterial viable counts was observed when a critical concentration of thymol was

reached, they proposed that the phenolic compounds sensitise the membrane and when active membrane sites are saturated there follows a sudden collapse of the cytoplasmic membrane and subsequent leakage of intracellular constituents. Carvacrol and eugenol are likely to have similar activity against bacterial cells. Ultee and co-workers found the activity of carvacrol to be dependent upon concentration, exposure time, pH and temperature, in a manner similar to other phenols. Considering these results and the hydrophobic nature of carvacrol, they proposed that the cellular target would be the cell membrane (Ultee et al, 1998). Other researchers have proposed that phenols present within plant essential oils may damage yeast cells by inhibiting enzyme pathways such as those involved in energy production and in the synthesis of structural components (Conner and Beauchat, 1984). However, no evidence was provided to support this hypothesis. In this thesis, it seems unlikely that interference with bacterial enzymes is the major cause of bacterial killing due to the short time period required for *B. cepacia* killing and because the bacterial cells do not need to be actively growing for killing to take place.

Transmission electron microscopy revealed cell debris and no intact cells only three minutes after addition of the minimum bactericidal concentration of thymol to 10^5 cfu/ml of the ET12 strain of *B. cepacia*. Such rapid destruction of cells is evidence to support the hypothesis that the phenolic compounds present within thyme oil attack the bacterial cell membrane causing leakage of intracellular components. Electron microscopy has also been employed by other researchers to elucidate the mode of action several oils against *E. coli*. Cox and co-workers (1998) observed that incubation of *E. coli* in the presence of tea tree oil caused loss of

cellular material, the coagulation of cytoplasmic constituents and the formation of blebs of material on the cells surface. Non-viable cells were observed to have intact cell membranes. Scanning electron microscopy employed in this thesis revealed cell debris and blebs of material on the surface of the limited numbers of intact bacterial cells remaining after two minutes incubation with thyme oil. In another study, Pattnaik and co-workers (1995) showed that *E. coli* cells did not lyse in the presence of oils of eucalyptus, lemongrass, peppermint and palmarosa oils. However, elongated, filamentous forms of *E. coli* were observed after cells were incubated in the presence of peppermint and palmarosa, but not eucalyptus or lemongrass oils, perhaps due to an effect on cell division or cell wall formation. In contrast, scanning electron microscopy employed in this thesis showed intact bacterial cells remaining after two minutes incubation in thyme oil to be shorter in appearance than control cells of *B. cepacia*.

Accumulated results suggest that different oils attack different bacteria in different ways, however damage to the cell membrane is a common observation.

In this thesis, time course experiments carried out on strains of *B. cepacia* in the presence of thyme oil or thymol showed rapid reduction in bacterial numbers, both in the presence and absence of a carbon source. Interestingly, the speed of killing of log phase *B. cepacia* cells was only slightly faster than that of stationary phase cultures. This rapid killing regardless of bacterial growth phase, supports the hypothesis that disruption of the *B. cepacia* membrane is responsible for cell death. The greatest time period from addition of thyme oil to killing of all *B. cepacia* cells was 30 minutes. The few cells remaining ten minutes after addition, may have

already been damaged by the phenols present within the oil, but recovered when removed from the antimicrobial agent and spread onto nutrient agar.

In conclusion, it seems reasonable to speculate that thyme oil, a mixture of the phenols thymol and carvacrol, kills cells of *B. cepacia* by disrupting the integrity of the cell membrane. Enzymes present within the cell membrane or the phospholipids themselves may also be damaged. Further investigations are required to clarify the precise mechanism(s) responsible for membrane disruption.

CHAPTER 6 ENDOGENOUS ANTIMICROBIAL PEPTIDES

6.1 Activity of hBD-1 against *P. aeruginosa* and *B. cepacia*.

To investigate activity of hBD-1 against *B. cepacia*, 5×10^4 cfu of an overnight cultures of non-mucoid *P. aeruginosa* (J1385) and three strains of *B. cepacia*, (the ET12 epidemic strain J2315, the environmental isolate J2421 and the proposed biological control strain J2742) were challenged with 50µg/ml of hBD-1 in the presence of NaCl concentrations between 0 and 150mM. Experiments were carried out in 10mM phosphate buffer containing 0.1% D-Glucose to investigate whether hBD-1 activity is salt sensitive. Duplicate reactions were prepared containing buffer alone or synthetic peptide rehydrated in buffer across the same range of NaCl concentrations. Each reaction was sampled in duplicate. The results are recorded in figures 6.1, 6.3, 6.4 and 6.5 below. SEM error bars are shown.

Figure 6.1 Activity of hBD-1 against *P. aeruginosa* (J1385) over a range of salt concentrations.

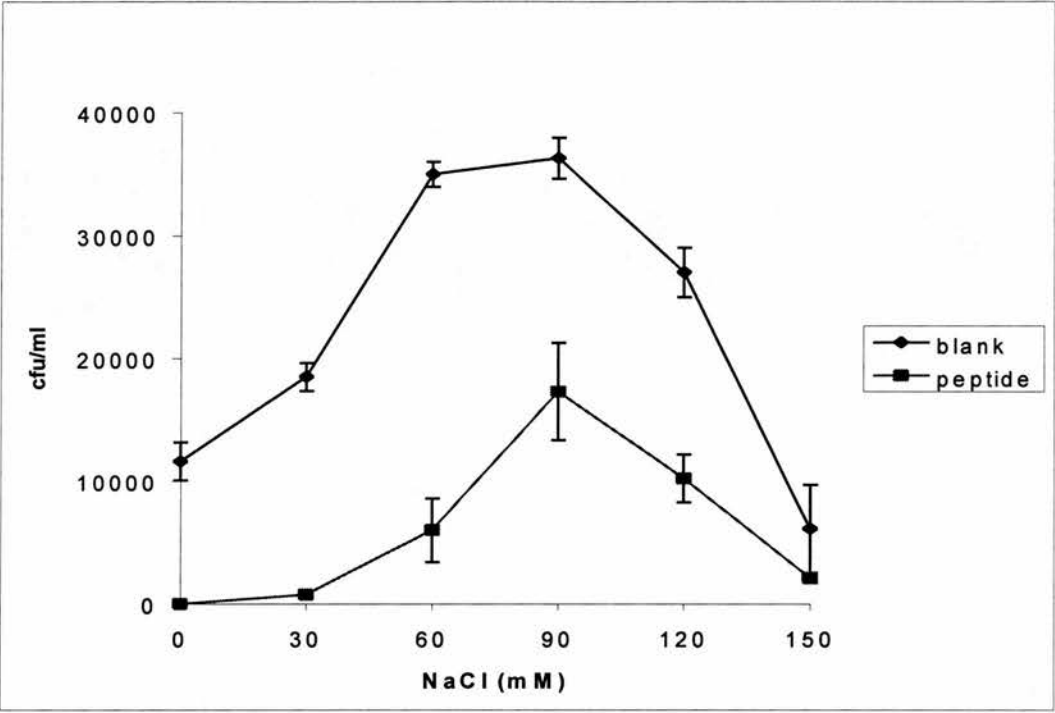


Figure 6.2 Percentage survival of *P. aeruginosa* when challenged with hBD-1 at a range of salt concentrations.

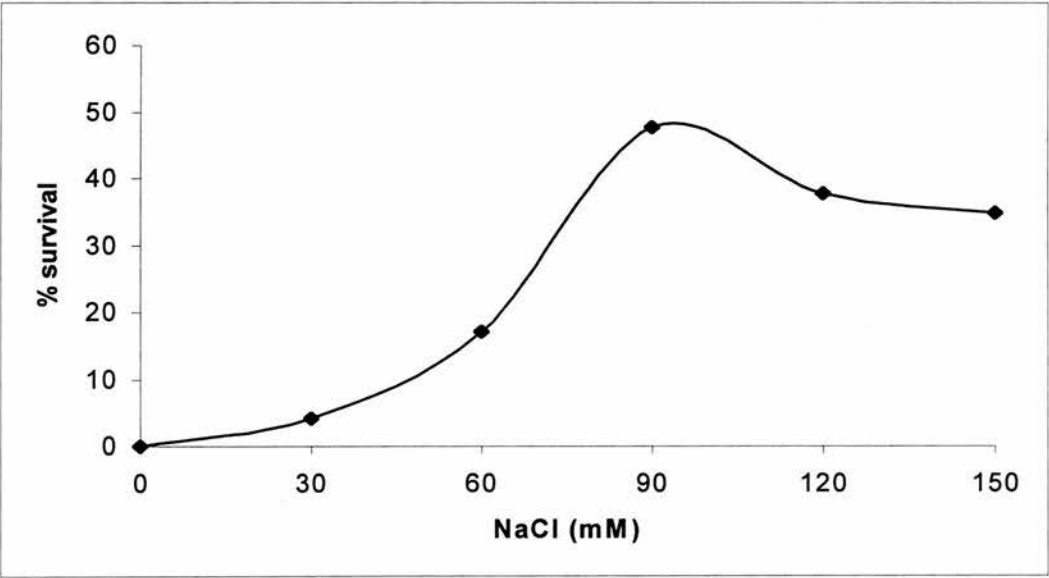


Figure 6.2 confirms that inhibitory activity of the hBD-1 peptide against *P. aeruginosa* is salt sensitive. Activity of the peptide was greatest in the absence of salt or at low salt concentrations up to 60mM. One hundred percent killing of *P. aeruginosa* was observed when cells were exposed to hBD-1 when no salt was present. Only 17% of the bacterial cells survived incubation with hBD-1 in 60mM NaCl for 20 minutes relative to the control. At higher salt concentrations however, activity of the peptide was considerably reduced. Approximately 40% of bacterial cells survived challenge with the peptide at salt concentrations of 90mM and above. Thus, the hBD-1 peptide was confirmed as having salt-sensitive antimicrobial activity against a non-mucoid clinical strain of *P. aeruginosa*.

Figure 6.3 Activity of hBD-1 against the ET12 strain of *B. cepacia* (J2315).

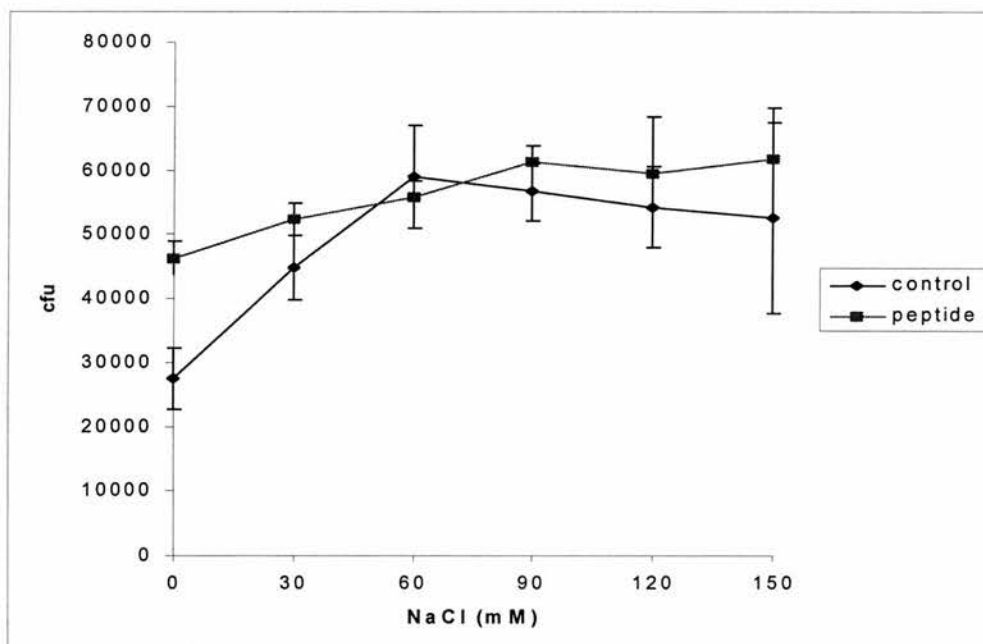


Figure 6.3 shows that hBD-1 does not inhibit the growth of the ET12 strain of *B. cepacia*. In fact at low salt concentrations the presence of the peptide may even enhance the growth of this strain. Due to the multivorous nature of this organism it is even possible that *B. cepacia* is capable of using the antimicrobial agent as a carbon source in a similar fashion to that previously reported for penicillin (Beckman and Lessie, 1979).

Figure 6.4 Activity of hBD-1 against an environmental strain of *B. cepacia* (J2421).

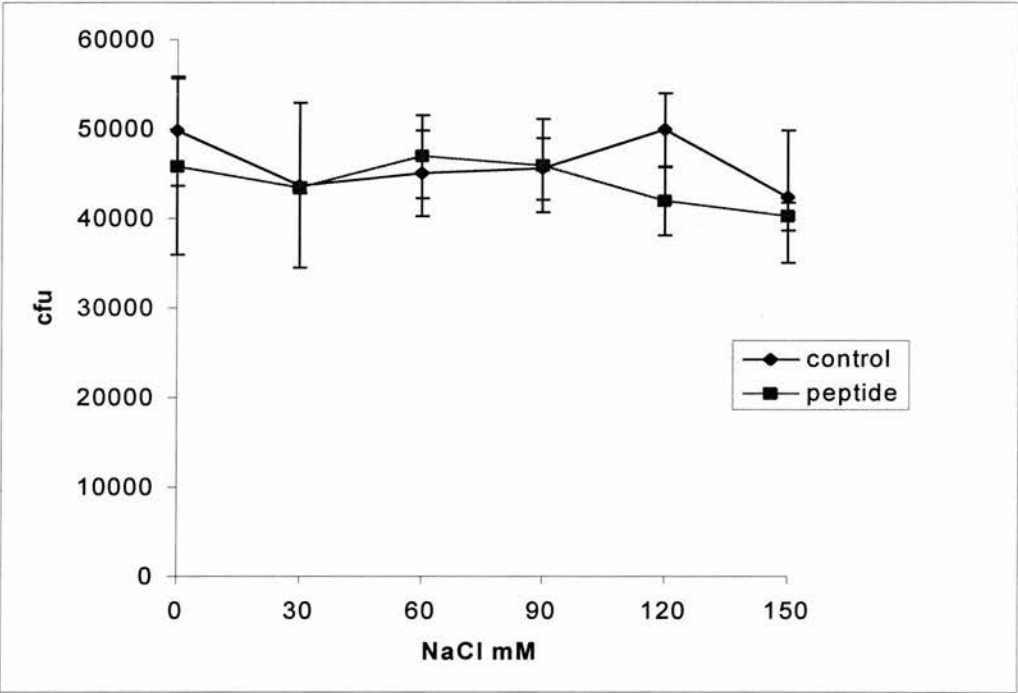
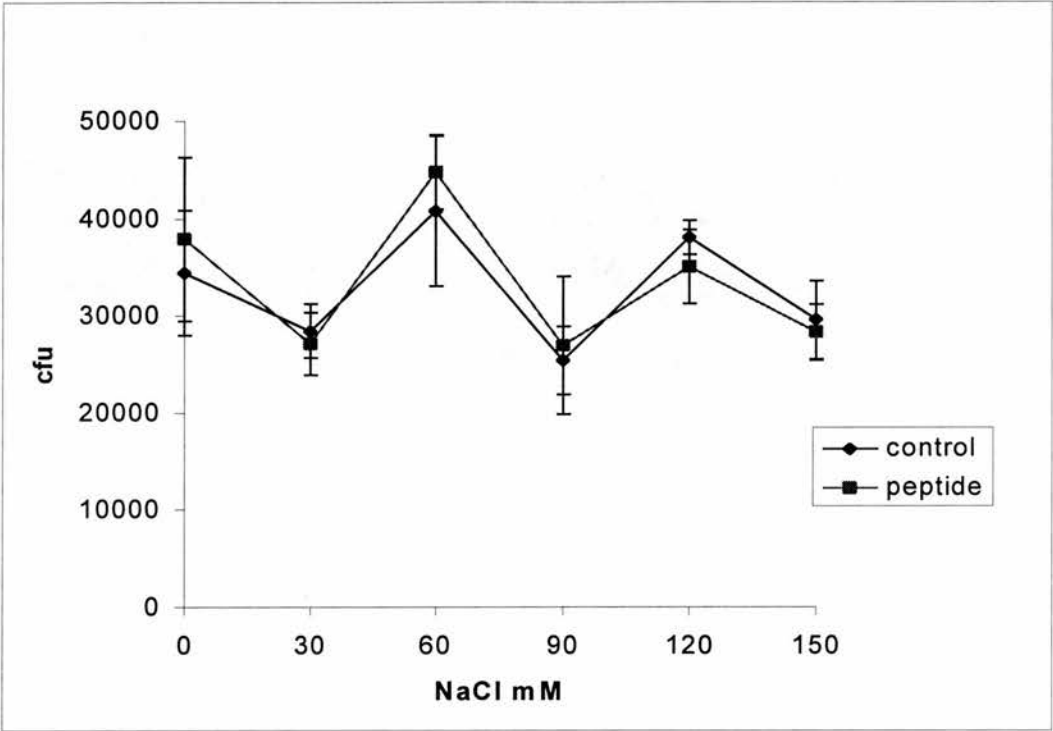


Figure 6.5 Activity of hBD-1 against the proposed environmental control strain of *B. cepacia* (J2742).

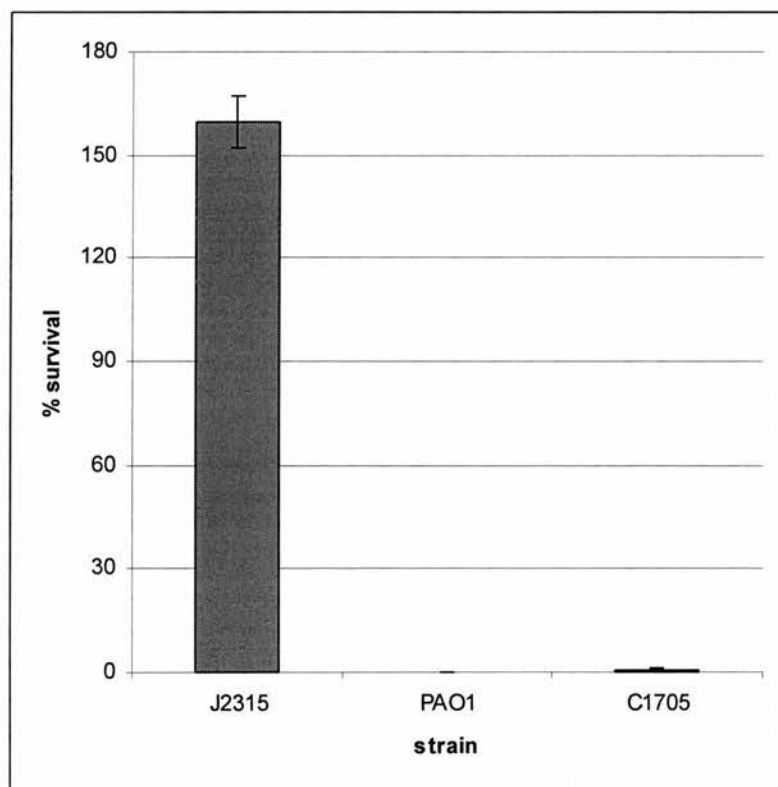


Both environmental strains of *B. cepacia* were also found to be resistant to the antimicrobial activity of hBD-1. Even at low salt concentrations, there was no difference between the numbers of viable bacterial cells remaining in reactions containing the peptide and the controls. Thus, none of the strains of *B. cepacia* tested showed susceptibility to hBD-1.

6.2 Antimicrobial activity of elafin

To determine whether the proteinase inhibitor elafin, had antimicrobial activity similar to the activity recently recognised in the other member of the ALP family, SLPI (Hiemstra et al, 1996), preliminary experiments were carried out in which 5×10^4 cfu/ml of *S. aureus*, *P. aeruginosa* and *B. cepacia* were challenged with recombinant adeno-elafin. Experiments were conducted in the absence of salt as the activity of this molecule is thought to be salt sensitive. Results shown represent the average of four separate experiments and are expressed as a percentage of controls which contained only salt-free phosphate buffer.

Figure 6.6 Survival of *B. cepacia* (J2315), *P. aeruginosa* (PAO1) and *S. aureus* (C1705) upon challenge with recombinant elafin.



The results in figure 6.6 show that recombinant elafin has antimicrobial activity against *P. aeruginosa* and *S. aureus*, but not *B. cepacia*. It was noted that survival of both *P. aeruginosa* and *S. aureus* was reduced after two hours incubation in the salt-free buffer even in the absence of elafin (data not shown). This may have been due to the absence of salt from this buffer or because only a single carbon source (D-glucose) was available. In contrast, *B. cepacia* appears to thrive in the presence of elafin and salt-free buffer. Again it is possible that *B. cepacia* is capable of using elafin as a carbon source.

6.3 Discussion: Endogenous antimicrobial peptides

6.3.1 Antimicrobial activity of hBD-1

Synthetic hBD-1, a mature peptide of 36 amino acids in length was shown to have salt sensitive antimicrobial activity against *P. aeruginosa*, but to have no inhibitory activity against *B. cepacia*. The murine homologue of hBD-1, mBD-1 also had no antimicrobial activity against *B. cepacia* (J2315) regardless of salt concentration (data not shown). Activity of the two known human β -defensins (hBD-1 and hBD-2) is considered to be salt sensitive (Goldman et al, 1997; Bals et al, 1998a). A sharp decrease in the antimicrobial activity of synthetic hBD-1 against *P. aeruginosa* was observed when salt concentrations increased from 50mM to 125mM (Goldman et al, 1997). Interestingly, at high salt concentrations (150mM), 50% of the antimicrobial activity of the peptide remained. The length of the synthetic peptide analysed in these studies was not stated. The 36 amino acid mature form of hBD-1 isolated from human urine has been reported to maintain virtually all its antimicrobial activity against *E. coli* in both dilute and normal urine, whereas longer synthetic forms of hBD-1 which included part of the pro-piece of the peptide exhibited drastically reduced activity in the presence of salt (Valore et al, 1998). Bals et al (1998a) reported that the activity of hBD-2 against *E. coli* was diminished eightfold when salt concentrations were increased from 20mM to 150mM.

The synthetic hBD-1 used in this thesis at a concentration of 0.05mg/ml retained almost half of its antimicrobial activity against *P. aeruginosa* at salt concentrations of 90mM and above, however the peptide was much more active at NaCl

concentrations of 60mM or less. In the absence of NaCl all bacterial cells were killed by the peptide. Other studies have confirmed the antimicrobial activity of hBD-1 against strains of *P. aeruginosa* at peptide concentrations between 60 and 500µg/ml (Goldman et al, 1997) and against *E. coli* at concentrations between 0.3 and 10µM (Valore et al, 1998).

In this thesis, despite confirmation that the peptide preparations were exhibiting activity against *P. aeruginosa*, no antibacterial activity was observed when *B. cepacia* strains were challenged with synthetic hBD-1 and mBD-1. The results presented in chapter 3, together with evidence from previous studies, indicate that *B. cepacia* is also inherently resistant to larger cationic molecules such as polymyxin and to aminoglycoside antibiotics (Fass and Barnishan 1980).

Polycationic antibiotics such as polymyxin and aminoglycosides interact with lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria. Divalent cations, such as Mg^{2+} and Ca^{2+} normally bind to LPS because of the polyanionic nature of this molecule. The integrity of the bacterial outer membrane is maintained as the divalent cations link adjacent LPS molecules. Polycationic antibiotics have greater affinity for LPS and thus competitively displace the native divalent cations. Once bound, these large molecules cause gaps to form in the outer membrane through which more of the antibiotic can flow. Polycationic antibiotics can then reach their final target, the cytoplasmic membrane, where these amphipathic molecules aggregate to form channels causing leakage of cytoplasmic components, and thus cell death (Hancock et al, 1984; 1995).

It appears that polycationic antibiotics are unable to permeate the outer membrane of *B. cepacia* allowing the organism to remain resistant to this class of antibiotics. A

link has been proposed between polymyxin resistance and the presence of a high content of phosphate-linked 4-amino-4-deoxyarabinose in the *B. cepacia*, *Proteus mirabilis* and *Chromobacterium violaceum* (Vaara, 1992).

A study of polymyxin B resistant mutants of *S. typhimurium* has shown that an increased level of 4-amino-4-deoxyarabinose bound to the lipid A phosphate of LPS would dispense with the necessity for divalent cations to form crossbridges between adjacent phosphate molecules. Instead, positively charged arabinose forms ion pairs with the negatively charged phosphates present on adjacent LPS molecules to provide outer membrane stability. Mutants of *P. mirabilis* which lack 4-amino-4-deoxyarabinose are sensitive to polymyxin (Kaca et al, 1990).

B. cepacia LPS has been shown to contain high levels of 4-amino-4-deoxyarabinose which could explain the resistance of this organism to EDTA and cationic antibiotics (Cox and Wilkinson, 1991). The low content of KDO and phosphate present in the LPS of *B. cepacia* also decreases the overall negative charge of this molecule when compared to other Gram-negative bacteria and thus further reduces the capacity of the outer membrane of *B. cepacia* to bind cations (Cox and Wilkinson, 1991).

Interestingly, these chemical characteristics of *B. cepacia* might explain the significant differences which have been described in the inflammatory properties of LPS from *B. cepacia* and *P. aeruginosa* (Shaw et al, 1995).

6.3.1 Antimicrobial activity of elafin

Elafin is also polycationic and the antimicrobial activity exhibited by this molecule may also come from an ability to disrupt bacterial membranes. Results from the preliminary experiments performed in this thesis show *P. aeruginosa* and *S. aureus* to be sensitive to the antimicrobial activity of this peptide, whereas *B. cepacia* appears resistant.

Unlike the synthetic peptide hBD-1, the recombinant adeno-elafin used in this thesis was synthesised within human cells (A549). Although these experiments provide useful preliminary data they were not well controlled and the possibility exists that some other compound present within the cell culture system was causing the antibacterial effect. Synthetic elafin was therefore synthesised and tested to exclude this possibility. Recently, full-length synthetic elafin has been shown to have antibacterial activity against *P. aeruginosa* (PAO1) and *S. aureus* (C1705) (Simpson et al, 1999).

As synthetic peptides are very expensive and difficult to produce, the small quantity that was available was used to study the antimicrobial activity of elafin against only one strain of each of *P. aeruginosa* and *S. aureus*, in detail. These strains were chosen as favourable results had been obtained with these organisms in the preliminary experiments. Due to the resistance of *B. cepacia* to elafin observed in the preliminary studies, the susceptibility of *B. cepacia* to synthetic elafin was not studied.

Mean results of between four and eight replicate experiments show that full length synthetic elafin killed significant numbers of PAO1 at doses of between 1 and 25 μ M.

The maximum effect of elafin against PAO1 was at 2.5 μ M, a concentration at which 93% of bacterial cells were killed. Killing of *S. aureus* by full length elafin increased with increasing concentrations of elafin and had its maximum effect at the highest concentration tested (25 μ M). At this concentration, 48% of the cells of *S. aureus* were killed. Experiments carried out using human serum albumin as a control showed that activity observed was not due to a non-specific peptide effect. If more synthetic elafin was to become available it would be interesting to determine that J2315 is resistant to the antimicrobial activity of this peptide. It would also be useful to study a range of *B. cepacia* strains to determine if the majority of strains of *B. cepacia* are resistant to the activity of this cationic peptide.

Problems were encountered in both the hBD-1 and elafin studies which may be due to the use of the salt-free phosphate buffer. Salt-free buffer was necessary for study of these cationic peptides as the activity of hBD-1 is salt sensitive, and perhaps that of elafin also. The detrimental effect of the buffer on both *P. aeruginosa* and *S. aureus* was more pronounced in the elafin studies, possibly due to the extended incubation times necessary in these experiments.

Differences may also be due to the different growth phases of the bacteria used in each experiment. Stationary phase bacterial cells as used in the defensin experiments may survive better in this buffer than log phase cell utilised in the elafin experiments. It may also be that the sudden transition from growth in rich media to the simple phosphate media with D-glucose as a sole carbon source has a detrimental effect upon bacterial survival. High levels of salt also appear to effect growth of *P. aeruginosa*. Physiological saline contains approximately 145mM NaCl, however,

the highest salt concentration used in the defensin studies had a slightly higher salt concentration of 150mM. Survival of *P. aeruginosa* was considerably diminished in the controls run alongside the defensin experiment carried out in phosphate buffer containing 150mM NaCl. However, it seems unlikely that this slight increase in salt concentration alone would so drastically affect the survival of bacterial cells. Thus, although both hBD-1 and elafin have considerable activity against individual strains of *P. aeruginosa* and *S. aureus*, neither were found to have antimicrobial activity against *B. cepacia*. Further studies are required but one explanation is that the outer membrane on *B. cepacia* is resistant to the action of polycationic antibiotics.

CHAPTER 7 CONCLUSIONS

The exploration of novel antimicrobial strategies against *B. cepacia* has proved necessary as this organism is inherently resistant to conventional antibiotics and poses a major threat to the health of immunocompromised patients, in particular those with CF. In this thesis, two main strategies are investigated, one is the study of antimicrobial compounds produced by plants and the other, an examination of the antimicrobial activity of cationic peptides present in human airway secretions. Examination of antimicrobials isolated from plants focused mainly on two plant species, garlic and thyme. Garlic contains the effective although unstable antimicrobial compound, allicin, which showed inhibitory activity against a range of *B. cepacia* isolates including strains such as the highly resistant and epidemic lineage ET12. Unfortunately, not only is allicin unstable, but it is also difficult to manufacture by chemical synthesis. Other compounds present in AGE were also revealed to possess antimicrobial activity against the ET12 strain of *B. cepacia*. In future, it may be useful to study the effect of ingestion of correctly dried garlic powder preparations on the antimicrobial activity of human serum. If antimicrobial activity associated with garlic consumption was observed in human serum it would be interesting to study potential beneficial effects in *B. cepacia* colonised CF patients. Allicin and some of the other compounds produced when garlic is cut or crushed are unlikely to survive the acidic conditions present in the stomach as alliinase is completely and irreversibly inhibited at a pH of 3.6 or lower. Thus, to allow allicin to be absorbed into the bloodstream, garlic powder preparations would require encapsulation as protection against low pH conditions of the stomach.

However, as allicin is thought to react in blood to form allylmercaptan, encapsulation may be unnecessary.

Thyme, in particular thyme oil, contains the antimicrobial compounds, thymol and carvacrol which are much more stable than allicin. However, antimicrobial use of these compounds presents other problems. As phenols, they have toxic properties if ingested or applied to mucus membranes including the lungs. However, relatively small concentrations of thyme oil were required for bactericidal activity against strains of *B. cepacia*, and these levels might be tolerated by the body without toxic effects. Even lower concentrations of thyme oil or its antimicrobial phenolic components, would be required if synergy occurred between these compounds and conventional antibiotics. It would therefore be fruitful to examine combinations of thyme oil and a variety of conventional antibiotics for synergistic antimicrobial activity against *B. cepacia*.

The endogenous antimicrobial peptides hBD-1 and elafin, like other previously studied cationic antibiotics, exhibit no inhibitory activity against *B. cepacia*. If the hypothesis is correct that high levels of salt in CF lung secretions deactivate antimicrobial peptides which would otherwise protect from bacterial infection, it would still not explain why *B. cepacia* is capable of causing human lung infection. The predilection of *B. cepacia* to colonise and infect the CF lungs must result from a defect in another aspect of lung defence. Further studies of the activity of synthetic human elafin against *B. cepacia* would be beneficial exclude the possibility that this compound has anti-*cepacia* activity.

There is an urgent need for the development of effective anti-*B. cepacia* agents as the organisms present 'resistance' deprives patients of effective therapy. Although the

administration of plant extracts may seem an unconventional approach to treatment, it may be a useful strategy to focus further research on the most potent plant extracts, namely those from thyme oil. Priority might be given to investigate potential synergistic activity between these compounds and conventional antibiotics.

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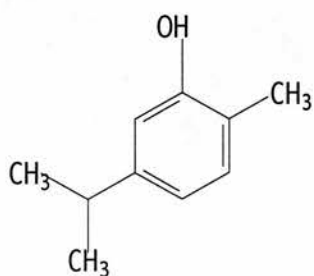
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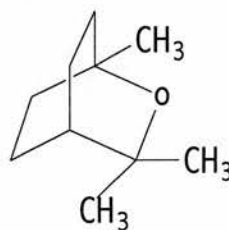
Appendix

Some chemicals found in plant essential oils

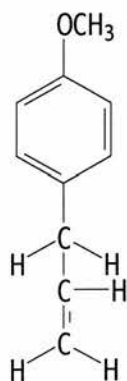
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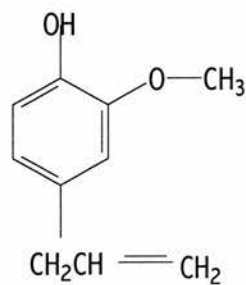
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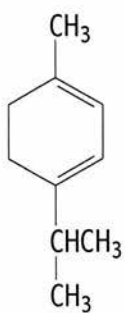
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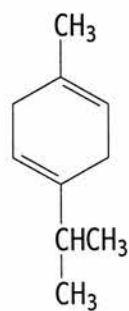
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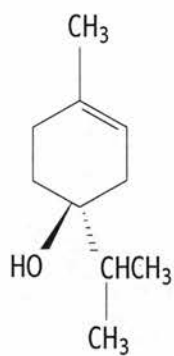
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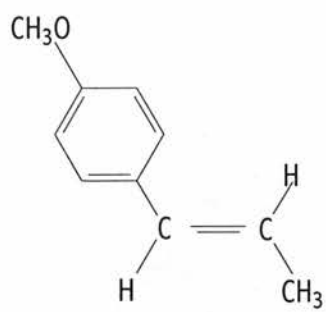
χ -terpinene



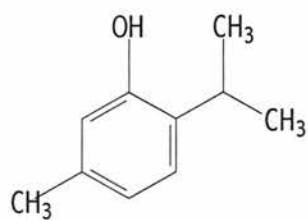
Terpinen-4-ol



Trans-anethole

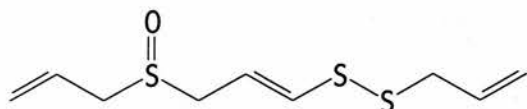


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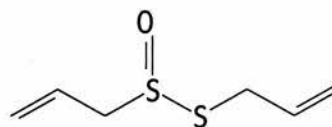


Sulphur containing compounds from garlic extracts

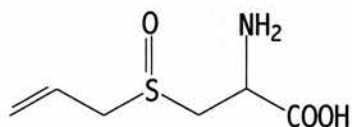
Ajoene



Allicin



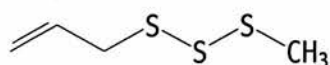
Alliin



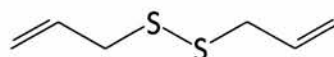
Allyl mercaptan



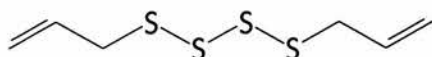
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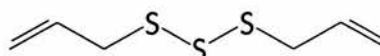
Diallyl disulphide



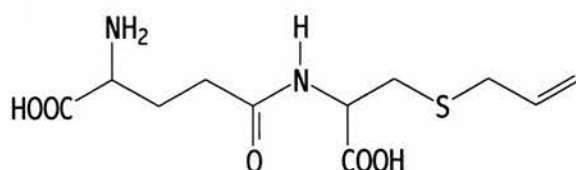
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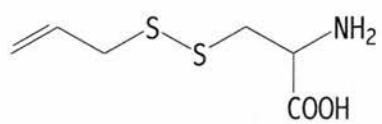
Diallyl trisulphide



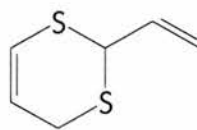
γ -glutamyl-S-allylcysteine



S-allylmercaptocysteine



Vinyldithiin

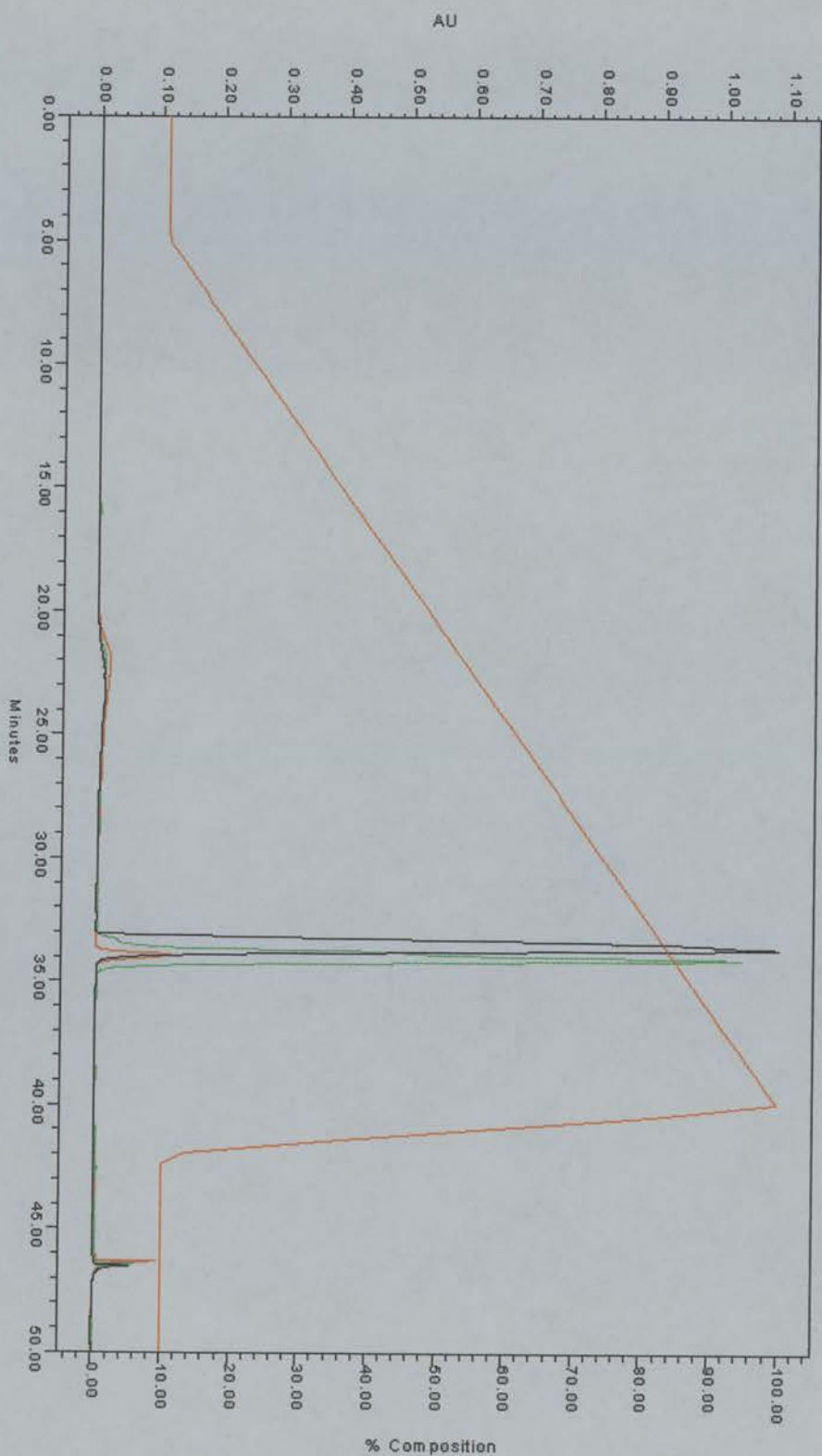


HPLC of carvacrol

Due to unforeseen circumstances, the HPLC analysis of carvacrol was carried out on alternative equipment. The alternative equipment comprised a Waters 600s controller (Milford, MASS, USA), Waters 626 pump and a Waters 486 detector, all controlled by Waters Millennium software. Separation was again performed on a continuous linear gradient of acetonitrile and detection was at 275nm. Thyme oil, thymol and carvacrol were all analysed with this alternative equipment so results could be compared.

Results show that each of the three samples produced an almost identical trace and thus could not be separated by RP-HPLC.

Analysis of thyme oil (green), thymol (red), and carvacrol (black) by RP-HPLC at 275nm



Mouse beta defensin-1 is a functional homolog of human beta defensin-1

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Abstract. Defensins are 3–4 kDa antimicrobial peptides of which three distinct families have been identified: α -defensins, β -defensins, and insect defensins. Recent investigations have shown that β -defensins are present in the human airways and may be relevant to the pathogenesis of cystic fibrosis (CF) lung disease. We report here the further characterization of a recently identified mouse β -defensin gene, *Defb1*, sometimes referred to as *mBD-1*, which is homologous to the human airway beta defensin *hBD-1*. We report that *Defb1* is expressed in a variety of tissues including the airways and, similar to *hBD-1*, is not upregulated by lipopolysaccharide (LPS). *Defb1* was found to consist of two small exons separated by a 16-kb intron and cytogenetic, and physical mapping linked it to the alpha defensin gene cluster on mouse Chromosome (Chr) 8. Functional studies demonstrate that, like *hBD-1*, *Defb1* demonstrates a salt-sensitive antimicrobial activity against *Pseudomonas aeruginosa*. Of relevance to CF lung disease is the fact that neither the *hBD-1* nor the *mBD-1* peptides are active against *Burkholderia cepacia*.

Defensins are a large family of peptides of which two groups exist in mammals: alpha defensins and beta defensins, which are distinguishable by the spacing and connectivity of the conserved cysteine residues within the mature peptides. It is thought that defensins function in the eradication of pathogens from the host system by inserting themselves into the bacterial membrane under the influence of membrane potential, forming channels which lead to leakage of cytoplasmic molecules and cell death (reviewed by Hancock 1997). Two bovine beta defensins, tracheal antimicrobial peptide (TAP; Diamond et al. 1991) and lingual antimicrobial peptide (LAP; Schonwetter et al. 1995), have been identified which are expressed from airway epithelial cells, and both were shown to have bactericidal activity against a broad array of organisms. These molecules were the only known airway epithelial defensins until recently when a human beta defensin molecule (*hBD-1*) was identified that was expressed in the airways (Goldman et al. 1997; McCray and Bentley 1997). Smith et al. (1996) demonstrated that the airway surface fluid (ASF) covering the apical surface of primary cultures of airway epithelial cells isolated from normal individuals had antimicrobial activity, but this activity was markedly reduced in ASF from epithelial cells from cystic fibrosis (CF) individuals. It had previously been reported that ASF had elevated concentrations of Cl^- and Na^+ in CF compared with normal (Joris et al. 1993; Gilljam et al. 1989), and Smith and associates (1996) demonstrated in vitro that when the salt concentration of normal ASF was increased to the CF level, the antimicrobial activity of the fluid was decreased. This led to the speculation that the molecule responsible for the antimicrobial activity in ASF

that was defective in CF might be a salt-sensitive defensin. An *hBD-1* synthetic peptide was shown to have broad spectrum bactericidal activity which was markedly reduced in salt concentrations similar to those reported for CF ASF, and it was, therefore, proposed that the reduced ability of *hBD-1* to kill bacteria in a CF environment was of relevance to the pathogenesis of CF lung disease. Although these recent findings are very encouraging and give some form of explanation for the early onset of bacterial colonization in the CF lung, they have so far been studied only in in vitro systems. Further functional studies of *hBD-1* with regard to its possible involvement in the onset of CF lung disease and the relevance of this defensin molecule in other infectious disorders may be facilitated by the use of a transgenic mouse model. Recently Huttner and colleagues (1997) described a mouse homolog of *hBD-1*. We confirm and extend these results to show the response of *Defb1* to LPS challenge and demonstrate the antimicrobial activity of synthetic *mBD-1* peptide.

Materials and methods

Expression of *Defb1*. Total RNA was isolated from a variety of tissues from C57/B16 mice using RNazol as described by the manufacturer. cDNA synthesis was accomplished using a 1st strand cDNA synthesis kit (Boehringer Mannheim) and the resultant cDNAs used as a template in a PCR reaction with primers *def1* (CCAGCTGCCCCATCTAATACC) and *def2* (AATCCATCGCTCGTCCTTTA) and the following conditions: denaturation at 94°C for 3 min; 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The amplified products (112 bp) were then analyzed on a 4% NuSieve agarose gel (FMC BioProducts) by electrophoresis. Reactions were verified for RNA amplification by including controls without reverse transcriptase.

LPS administration and semi-quantitative RT-PCR. 40 μg of LPS (*E. coli* 055:B5, Sigma) was administered to mice by intratracheal delivery, and the mice were sacrificed at various time points after instillation. Total RNA was isolated from the lungs and trachea, and RT-PCR for *Defb1* was performed as described above except with the primer *def5'* (CACTCTG-GACCTGGCTGCC) instead of *def1* which gave an amplified product of 277 bp. Amplification of *Hprt* from exon 3 to exon 8 was carried out in parallel to monitor the amount of input RNA with the forward CTGTA-GATTTTATCAGACTGAAGAG and reverse GTCAAGGGCATATC-CAACAACAAA primers and the following conditions: denaturation 94°C for 3 min; 30 cycles of 93°C for 30 s, 55°C for 1 min, and 75°C for 1.5 min. The amplified products were analyzed on a 1% agarose gel by electrophoresis.

Screening of the genomic libraries. A mouse BAC library from the cell line CJ7 derived from a mouse 129SV strain (Research Genetics Inc) was screened for *Defb1* by PCR using the primers *def1* and *def2* and conditions mentioned previously. Two plate pools, 421 and 524, which gave the expected PCR product, were then screened by hybridization with

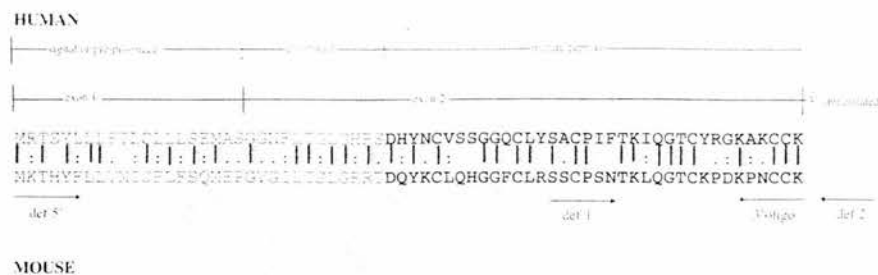


Fig. 1. Comparison of the human and mouse beta defensin peptide sequence. Vertical line, direct match; two dots, close similarity; one dot, some similarity. The peptide sequences used to produce the synthetic peptides are highlighted in bold. The positions of the oligos used for PCR and hybridization are also shown.

the entire cDNA sequence of *Defb1* as a probe. A mouse lambda GET library was also screened by hybridization, and positive clones were converted to plasmid as described (Nehls et al. 1994).

Characterization of genomic clones. Restriction enzyme analysis was performed on the BAC and lambda clones with a variety of 6-bp cutters. Probes corresponding to the 5' end of the cDNA (CACTCTGGACCCTG-GCTGCC) and the 3' end of the cDNA (ATTCTCTGTTAGAAGAGC) were used to map genomic fragments.

Co-localization of mouse alpha and beta defensins. BACs I8 and I20 were screened for the presence of alpha defensins with the probes TCCAGGCTGATCCTATCC and TGDATGCTATTGTAGAT, which are derived from the consensus regions, codon 17–23 and codon 62–68, which are found in all 16 mouse alpha defensins (Huttner et al. 1994).

Locus mapping. The BAC clones I8 and I20 and pDef1 were used as probes either in isolation or in combination for one- or two-color chromosome mapping. Fluorescence in situ hybridization (FISH) was performed according to previously published protocols (Fantes et al. 1995) with E14 IV mouse embryonic cells for the chromosome spreads. A chromosome-specific paint was used to confirm the identity of mouse Chr 8, and Dapi staining was used to reveal chromosomal banding patterns, which allowed the localization of the hybridization signal to a specific chromosomal region.

Peptide synthesis. hBD-1 was synthesized from Fmoc-Lys(Boc) loaded Wang resin (0.25 mmol), and mBD-1 was synthesized from Fmoc-Ser loaded Wang resin (0.48 mmol). 1 mmol of the amino acids was single coupled via HOCt/DIC with the exception of His, which was coupled with 3 mmol of HOCt. The hBD-1 peptide was cleaved from the resin by stirring in EDT (0.25 ml), thioanisole (0.5 ml), H₂O (0.5 ml) and phenol (0.75 g) for 2 min, then adding TFA (9.5 ml) and stirring for 4 h at room temperature. The resin was removed by filtration into ether from which the peptide was precipitated, washed with ether, and applied to a Vydac C18 column (flow rate 5 ml/min, 5 ml loop, 10–50% B over 28 min). mBD-1 was cleaved from the resin by stirring in EDT (0.25 ml) and H₂O (0.5 ml) for 2 min before adding TFA (9.5 ml). The mixture was stirred at room temperature under nitrogen for 2 h before being filtered into ether from which the cleaved peptide was precipitated, washed in ether, and taken into aqueous acetonitrile and lyophilized. The peptide was then applied to a Vydac C18 column (flow rate 8 ml/min, 5 ml loop, 10–90% B, 40 min). The purified peptides were analyzed by RP HPLC (Vydac C18, 5 min loop), R_f = 17 min, which, in both cases, resulted in a single peak, and MALDI TOF MS analysis showed the correct molecular weight for hBD-1 and mBD-1.

Functional analysis of synthetic mBD-1 and hBD-1. The synthetic peptides were tested against two strains of *P. aeruginosa*, lab strain PAO1 and CF clinical isolate J1385, and *B. cepacia* CF clinical isolate J2315. These clinical isolates were carefully chosen because of their pedigrees (Govan et al. 1993, 1996). Fresh, overnight cultures of bacteria were grown in nutrient broth and then suspended in phosphate-buffered saline at 1×10^9 colony forming units per ml. These samples were then split, resuspended and diluted in 10 mM phosphate buffer containing 0.1% D-glucose and a range of concentrations of NaCl (buffer pH 7.60 at 0 mM NaCl, pH 7.40 at 30 mM NaCl, pH 7.33 at 60 mM NaCl, pH 7.27 at 90 mM NaCl, pH 7.23 at 120 mM NaCl, and pH 7.20 at 150 mM NaCl). Two sets of duplicate

500- μ l reactions were prepared containing either buffer alone or synthetic peptide rehydrated in buffer, across the same range of NaCl concentrations, to which 5×10^4 bacteria in the appropriate salt concentration were added. These reactions were incubated for 20 min at 37°C, after which duplicate sets of serial dilutions were prepared from each sample, in buffer of the appropriate salt concentration, plated out on *Pseudomonas* Isolation agar (DIFCO) and incubated overnight at 37°C or on *Cepacia* Medium (Mast Diagnostics) and incubated at 37°C for 48 h and colony counts performed. The antimicrobial activity of the peptide was compared against buffer alone, to control for the effects on the bacteria of varying the NaCl environment.

Statistical analysis. The data generated were regarded as having a Poisson distribution; thus, a square root transformation was appropriate to homogenize the variance between groups. Analysis of the significance of differences between peptide and control samples was performed and illustrated graphically, plotting the NaCl concentrations against means under square root transformation, derived from the mean square error, with group standard errors. The means of the colony forming unit counts were used to calculate the proportion of bacteria surviving in the peptide-treated samples as a percentage of the counts from the buffer-only samples, to control for the effects of changes in NaCl concentration alone. These percentages were expressed as "percentage killing"; standard errors were estimated and displayed graphically.

Results

Identification of murine sequences homologous to hBD-1. Seven near identical cDNA sequences from mouse heart, kidney, embryonic carcinoma, and macrophage cDNA libraries were retrieved from an EST database following a BLAST search using the hBD-1 sequence (GenBank #AA104376, AA065510, AA071757, AA105324, AA107538, AA107977, AA108061). The peptide translation of these sequences was found to be 53% similar to hBD-1 (Fig. 1).

Genomic characterization of the *Defb1* gene. Two genomic clones of *Defb1*, I8 from plate 421 and I20 from plate 524, were isolated following the BAC library screen. Two clones were isolated from the lambda GET library which were subsequently converted into plasmids and shown to be identical; only one was used in subsequent analysis and was termed pDef1. Southern blot hybridization results indicated that both the I8 and I20 BAC clones contained the entire *Defb1* gene sequence, which was found to extend over approximately 22 kb (Fig. 2), although they were not identical as they were flanked by differing lengths of genomic sequence 5' and 3' to the gene. *Defb1* was found to consist of two exons separated by an intron of approximately 16 kb (data not shown). pDef1 was found to contain only exon two and approximately 5 kb of intronic sequence.

Chromosomal location of *Defb1* by FISH. By use of FISH, both the BACs and pDef1 were localized to band A4 of mouse Chr 8 (Fig. 3A–C). When the BACs were hybridized together, on interphase cells it was possible to see a region of overlap between the

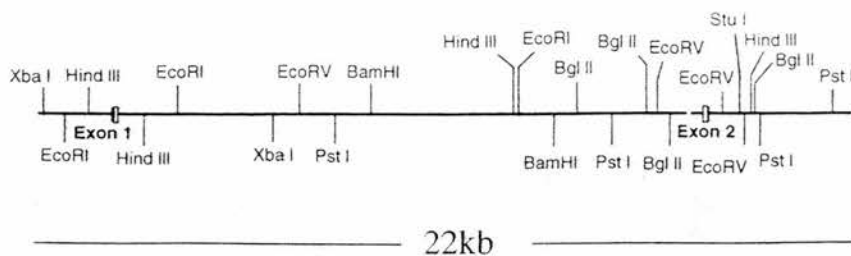
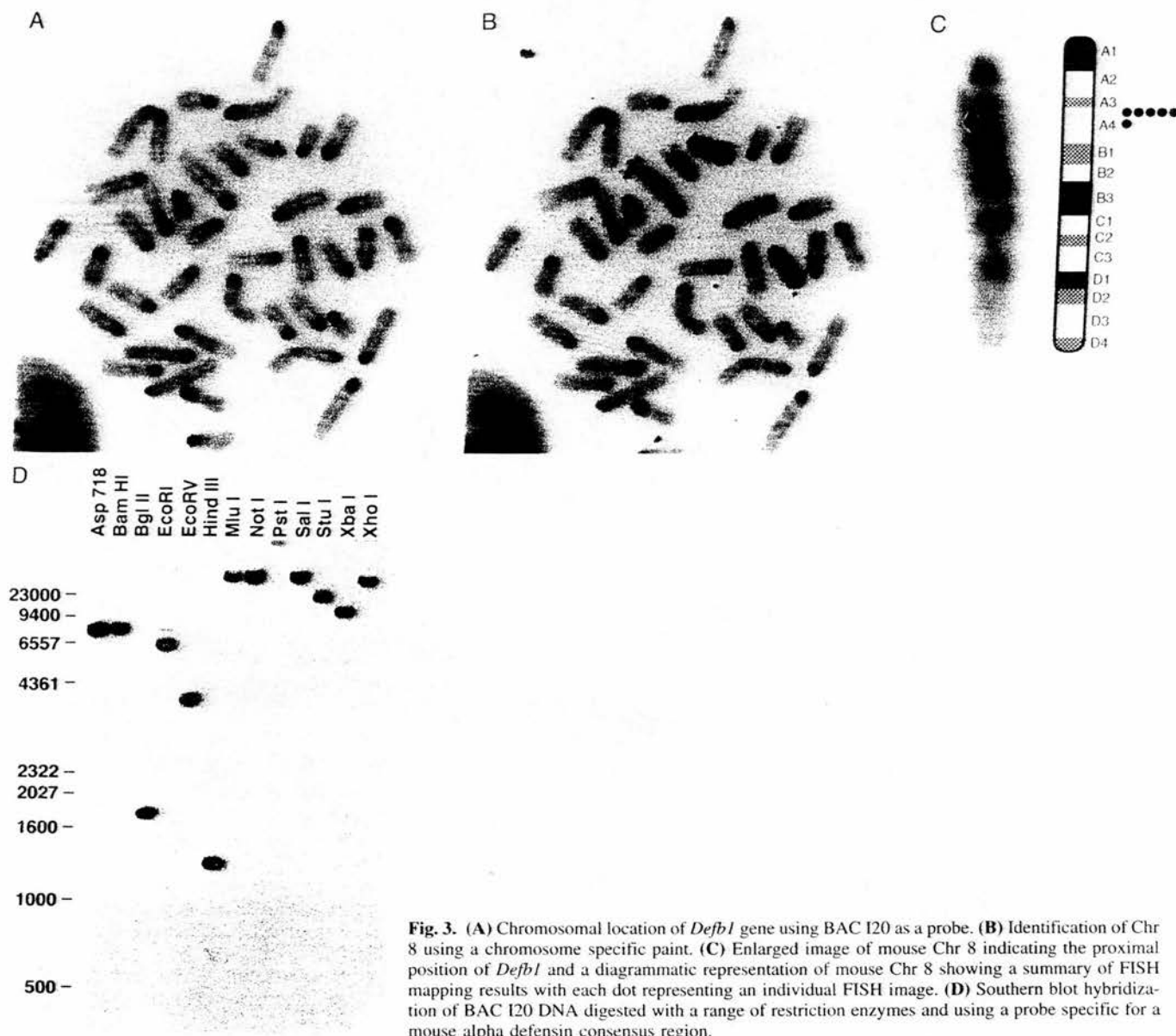
Fig. 2. Restriction map of *Defb1*.

Fig. 3. (A) Chromosomal location of *Defb1* gene using BAC I20 as a probe. (B) Identification of Chr 8 using a chromosome specific paint. (C) Enlarged image of mouse Chr 8 indicating the proximal position of *Defb1* and a diagrammatic representation of mouse Chr 8 showing a summary of FISH mapping results with each dot representing an individual FISH image. (D) Southern blot hybridization of BAC I20 DNA digested with a range of restriction enzymes and using a probe specific for a mouse alpha defensin consensus region.

BACs and also areas to either side to which only one BAC hybridized (data not shown).

Identification of alpha defensin sequence on BAC I20. Southern blot analysis of I8 and I20 with two distinct probes based on the alpha defensin consensus regions gave a positive signal only in BAC I20 (Fig. 3D). It was concluded that BAC I20 extended into the region of Chr 8 where the alpha defensin gene family is located, and therefore the α and β family of genes in the mouse are

separated by less than 80 kb. This is of interest because the human α and β defensin genes have also been shown to be located in close proximity to each other on a conserved syntenic region of human Chr 8.

Expression of *Defb1*. Expression of *Defb1* by RT-PCR was detected in the following tissues: kidney, lung, heart, gut, and purified alveolar macrophages (data not shown). We examined the response to LPS and did not see any evidence for gross upregu-

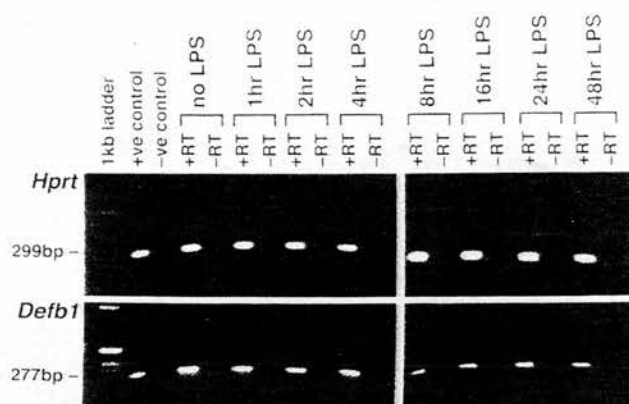


Fig. 4. RT-PCR of *Hprt* and *Defb1* from tracheal RNA following LPS instillation. The hours shown represent the time after LPS instillation when the mice were sacrificed with a plus (+RT) and minus (-RT) reverse transcriptase reaction shown for every sample.

lation of *Defb1* at any time point tested following administration directly to the mouse lung (Fig. 4).

Synthetic mBD-1 demonstrates a salt-sensitive antimicrobial activity. Using the synthetic hBD-1 peptide, we demonstrated a statistically significant killing effect against both strains of *P. aeruginosa* at a peptide concentration of 50 µg/ml (Fig. 5A–B, PAOI data not shown). This effect was shown to be extremely salt sensitive, dropping from 100% to 50% as the NaCl concentration rose from 0 mM to 90 mM, then leveling off at higher concentrations. In parallel studies using the synthetic mBD-1 peptide we observed a similar, statistically significant killing effect, against both strains of *P. aeruginosa*, at a peptide concentration of 500 µg/ml (but not significant at 50 µg/ml or 100 µg/ml) (Fig. 5C–D, PAOI data not shown). This antimicrobial effect displayed the same pattern of salt sensitivity, but was somewhat less effective than the synthetic hBD-1 peptide under optimal conditions, killing only 85% of the bacteria at 0 mM NaCl concentration. Thus, both synthetic peptides showed a rapid decline in activity between the low salt levels, thought to be physiologically normal in human airway surface fluid, and the raised NaCl environment thought to exist in the human CF lung, at which marked inhibition was observed.

We used the same assay to examine the effect of the peptides on *B. cepacia*. In these studies we observed no significant killing effect at any NaCl concentration with either peptide, under the conditions observed in our previous studies to be optimal for killing of *P. aeruginosa* (Fig. 5E–F). Surprisingly, at 0 mM NaCl, *B. cepacia* appeared to thrive in the presence of synthetic hBD-1 when compared with buffer, and it may be of interest that previous studies have shown that *B. cepacia* can use other antibacterial substances to its advantage such as penicillin G as its sole source of carbon and energy (Beckman and Lessie 1979).

Discussion

Defensins have been shown to be a widespread and ancient means of host defense existing as two distinct families in mammals. Recently, Liu and coworkers (1997) concluded that the α and β defensins are derived from a common ancestral gene because of the close proximity of the *hBD-1* to the human α gene cluster. Both alpha and beta defensin gene members have been mapped to human Chr 8p23.1-23.2 (Liu et al. 1997). The mouse alpha defensin genes lie in a cluster on the proximal region of mouse Chr 8, and Huttner and associates (1997) mapped *Defb1* locus to the same region of mouse Chr 8 by analysis of DNA from two sets of multilocus crosses. We confirm the map position of *Defb1* to mouse Chr 8 region A4 and physically show that the alpha defen-

sin gene cluster lies within 80 kb. The close proximity of these two gene families and similarity of the beta genes between species (53% at the amino acid level) compared with the low level of similarity between alpha and beta genes within a species strongly suggests that gene duplication of a common ancestral defensin gene occurred before human mouse species divergence.

A heightened salt concentration of CF ASF has been implicated in the decreased ability of hBD-1 to destroy bacteria (Smith et al. 1996) and therefore it was of interest to find out whether the mBD-1 peptide had the same antimicrobial activity. CF is characterized by its progression from repeated childhood infections with a relatively restricted range of organisms including *Staphylococcus aureus* and *Haemophilus influenzae* to chronic colonization with *P. aeruginosa* and the additional impact of *B. cepacia* infection. We have demonstrated that the synthetic mBD-1 peptide displays salt-sensitive antibacterial activity against *P. aeruginosa* similar to that of hBD-1. We have also demonstrated an absence of antibacterial activity when using the synthetic peptides against *B. cepacia*. Our observations support previous suggestions that this organism may be resistant to cationic peptides, and we conclude that the predisposition of CF patients to pulmonary infection with *B. cepacia* results from impairment of some other component of lung defense.

The similarity of the results obtained with the human and mouse peptide suggests that a comparable lung defense mechanism is present in the mouse, which may be impaired by abnormalities in the ionic composition of the airway surface fluid, although, to date, no measurements of the ionic composition of the ASF in normal and CF mice are available. Our results show that synthetic mBD-1 is required at a much higher concentration than synthetic hBD-1 to achieve antibacterial activity. This may reflect differences in the success of peptide synthesis or, alternatively, it is possible that these molecules possess slightly different profiles in vivo, in terms of the extent of their effects, the pathogens against which they act, and their dependence upon the synergistic effects of other components of the defense mechanism or their relative importance within it.

The discovery of a second human beta defensin (hBD-2) expressed in the airways and upregulated by exposure to LPS (Harder et al. 1997a) and also located in the same chromosomal region as hBD-1 (Harder et al. 1997b) suggests it is possible that additional airway defensins are also present in the mouse. Recent studies demonstrating a salt-sensitive synergistic antibacterial relationship between the ASF components, lysozyme and lactoferrin (Singh and Welsh 1997), suggest a complex array of interacting antimicrobial factors contributing to an innate lung defense system. However, it is obvious that CF lung disease is more complex in its etiology than simply a perturbation of the antimicrobial components of the airway surface fluid. Indeed, the partial inhibition of such broad-spectrum antibacterial agents would not be expected to result in the very specific profile of infections evident in CF. The pathogenesis of CF lung disease will no doubt be the end product of multiple abnormalities downstream from the mutation of *CFTR*.

In summary, we have demonstrated the existence of a mouse beta defensin, *Defb1*, which is expressed in the airways, but is not upregulated by LPS exposure and, like hBD-1, displays salt-sensitive antibacterial activity against the major CF-associated pathogen *P. aeruginosa*, but not against *B. cepacia*. These observations justify the use of the mouse as a model system to study the contribution of defensins to host innate defense and their relevance to CF lung disease and other infectious disorders.

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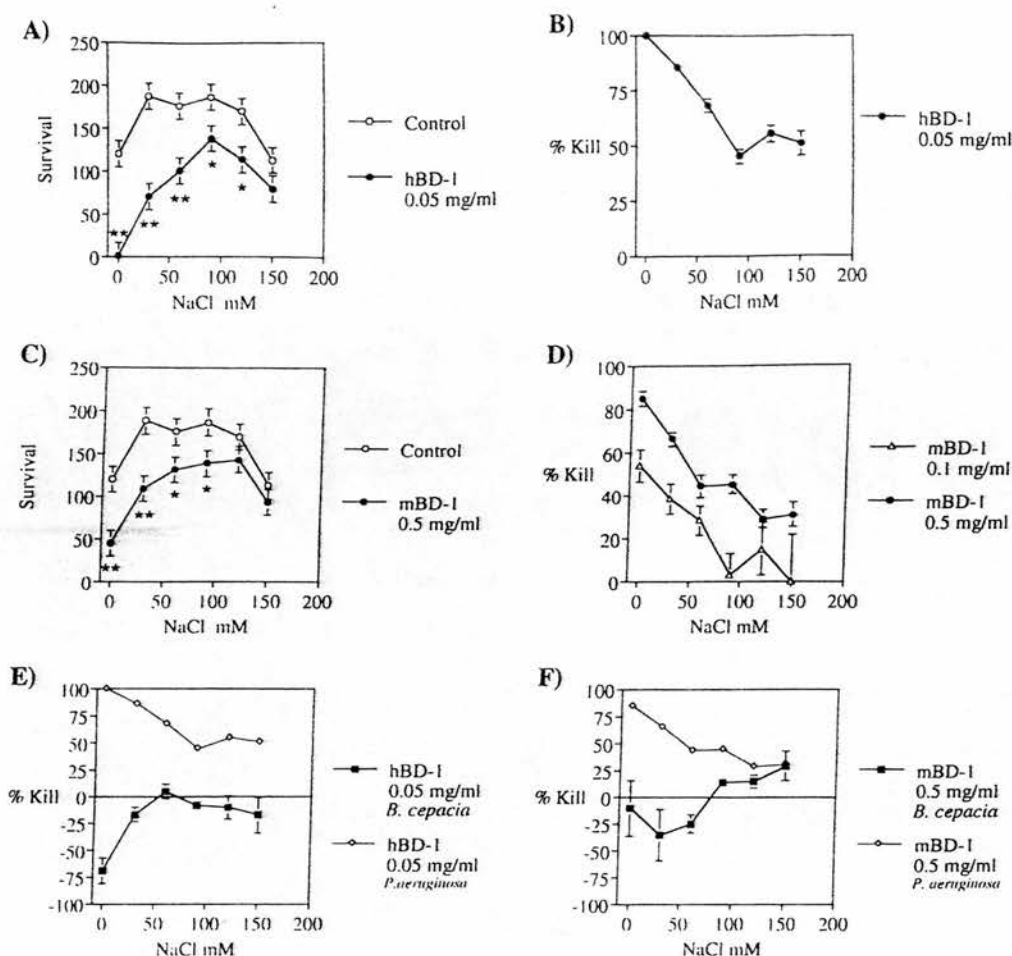


Fig. 5. Functional analysis of synthetic hBD-1 and mBD-1. *P. aeruginosa* J1385 was treated with hBD-1 at 0.05 mg/ml (A–B), mBD-1 at 0.5 mg/ml (C–D), or 0.1 mg/ml (D) or buffer alone. *B. cepacia* J2315 was treated with hBD-1 at 0.05 mg/ml (E), mBD-1 at 0.5 mg/ml (F) or buffer alone. NaCl concentrations were plotted against colony forming unit count means under square root transformation to allow statistical analysis (A and C) and against the percentage of bacteria killed in the peptide-treated sample, in comparison with control, using means of colony forming unit counts (B, D, E and F). At each point $n = 4$, standard error bars have been applied to all points. Two asterisks indicate significance at the 1% level, one asterisk indicates significance at the 5% level. These graphs are representative of repeated experiments.

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Elafin (elastase-specific inhibitor) has anti-microbial activity against Gram-positive and Gram-negative respiratory pathogens

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Abstract Elafin (elastase-specific inhibitor) is a low molecular weight inhibitor of neutrophil elastase which is secreted in the lung. Using synthetic peptides corresponding to full-length elafin (H₂N-¹AVT.....⁹⁵Q-OH), the NH₂-terminal domain (H₂N-¹AVT.....⁵⁰K-OH) and the COOH-terminal domain (H₂N-⁵¹PGS.....⁹⁵Q-OH), we demonstrate that elafin's anti-elastase activity resides exclusively in the COOH-terminus. Several characteristics of elafin suggest potential anti-microbial activity. The anti-microbial activity of elafin, and of its two structural domains, was tested against the respiratory pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Elafin killed both bacteria efficiently, with 93% killing of *P. aeruginosa* by 2.5 μM elafin and 48% killing of *S. aureus* by 25 μM elafin. For both organisms, full-length elafin was required to optimise bacterial killing. These findings represent the first demonstration of co-existent anti-proteolytic and anti-microbial functions for elafin.

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Key words: Elafin; Human neutrophil elastase; Anti-microbial activity; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Introduction

As part of the immediate defence against bacterial pathogens, the lung is equipped with a number of endogenous anti-microbial peptides, including defensins, secretory leucocyte protease inhibitor (SLPI), lysozyme and lactoferrin [1–6]. In addition, bacteria stimulate the recruitment and activation of neutrophils, resulting in engulfment of organisms and ultimately their destruction by oxidants or proteolytic enzymes such as human neutrophil elastase (HNE) [7]. Natural inhibitors of HNE in the lung are thought to play a critical role in neutralising HNE liberated extracellularly, thus circumventing proteolytic damage to the host [8].

Elafin (also known as elafin-specific inhibitor (ESI) or skin anti-leucoprotease (SKALP)) is a low molecular weight (9.9 kDa) inhibitor of HNE and proteinase 3, which is secreted in the respiratory tract [9,10]. Along with α_1 -protease inhibitor (α_1 -PI) [8] and SLPI [11], elafin comprises an integral part of the 'anti-elastase shield' in the lung [12].

Several features suggest an additional anti-microbial function for elafin. In particular, elafin is highly cationic, it is

expressed selectively in the lung, the skin and at mucosal surfaces and SLPI (which has 42% sequence homology with elafin) has anti-microbial activity [5,13–17].

In this study, we describe an investigation of the anti-microbial activity of elafin against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two important pulmonary pathogens with a particular propensity to antibiotic resistance [18–23].

2. Materials and methods

2.1. Proteins

Elafin peptides were produced synthetically in accordance with the derivation of amino acids from the established gene sequence [13] and were supplied by Albachem (Edinburgh, UK), using standardised protocols described elsewhere [24]. Three peptides were provided, namely full-length elafin (H₂N-¹AVT.....⁹⁵Q-OH), the NH₂-terminal domain (H₂N-¹AVT.....⁵⁰K-OH) and the COOH-terminal domain (H₂N-⁵¹PGS.....⁹⁵Q-OH). The terminal amino acid of the NH₂ domain and the first amino acid of the COOH domain were determined by analysis of the established crystal structure of a 57 amino acid fragment of elafin (H₂N-³⁹AQE.....⁹⁵Q-OH) [17], in conjunction with the established sequence of human SLPI, which has 42% sequence homology with elafin [13–15,25]. The molecular weights of elafin moieties, determined by mass spectrometry (Albachem, Edinburgh, UK) were 9925 Da for full-length elafin, 5172 Da for the NH₂-terminal domain and 4776 Da for the COOH-terminal domain.

Human serum albumin (HSA) was purchased from Sigma Chemicals (St. Louis, MO, USA). Recombinant human SLPI was purchased from R and D Systems (Minneapolis, MN, USA). Lyophilised preparations of elafin, SLPI and HSA were all reconstituted in 0.01 M K₂HPO₄/KH₂PO₄, pH 7.4 (phosphate buffer).

2.2. Bacteria

PAO1, a clinical strain and well-characterised type strain of *P. aeruginosa*, and C1705, a clinical strain of *S. aureus*, were available in-house [20,24].

2.3. HNE activity assay

The HNE activity assay has been described in detail elsewhere [26]. Briefly, serial dilutions of test inhibitor were added to 300 ng of purified HNE (Elastin Products, Owensville, MO, USA). All dilutions were performed in Tris 50 mM, Triton 0.1%, sodium chloride 0.5 M, pH 8.0. The samples were incubated for 15 min at 37°C before addition of the chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma). In positive controls, buffer replaced test inhibitor. The change in absorbance, measured spectrophotometrically at 405 nm (MR5000 Plate Reader, Dynatech, Dynex, Billingham, UK), was expressed as a function of time. The inhibitory capacity of test inhibitors was derived by extrapolation to the ordinate of curves obtained by plotting the HNE activity against the concentration of inhibitor added, as described elsewhere [27].

2.4. Assay of microbial activity

Bacteria were grown initially as colonies on Columbia agar (Unipath, Basingstoke, UK) and, then, in 10 ml tryptone soya broth (TSB) (Unipath) overnight at 37°C in an orbital shaker (Gallenkamp, Fisher Scientific, Loughborough, UK) at 200 rev/min. 100 μl of the bacterial culture was resuspended in 10 ml fresh TSB and incubated for 3 h at 37°C with rotation, corresponding to a point compatible with logarithmic growth for both PAO1 and C1705. The resulting suspension

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Abbreviations: HNE, human neutrophil elastase; α_1 -PI, α_1 -protease inhibitor; SLPI, secretory leucocyte protease inhibitor; HSA, human serum albumin; TSB, tryptone soya broth; LPS, lipopolysaccharide; CF, cystic fibrosis

was centrifuged at 4500 rpm for 20 min at room temperature (Bio-fuge, Heraeus Instruments, Kendro, Bishops Cleeve, UK), the supernatant discarded and replaced with 10 ml phosphate buffer and then resuspended. This process was repeated once. The quantity of viable bacteria was calculated from pre-constructed growth curves and dilutions made in phosphate buffer to give an estimated count of 5×10^4 viable colonies per ml.

In experiments using purified elafin, HSA or SLPI, 30 μ l aliquots of the bacterial suspension were added to 90 μ l aliquots of test substance, each diluted in phosphate buffer to give final concentrations of 1, 2.5, 10 or 25 μ M of the test substance. In positive controls, 90 μ l of phosphate buffer replaced the test solution and in negative controls, 30 μ l of phosphate buffer replaced the bacterial suspension.

The test bacterial suspension mix was incubated for 2 h at 37°C. Appropriate dilutions were made in phosphate buffer and 100 μ l aliquots plated out on Columbia agar. Colonies were counted after incubation of the plates for 16 h at 37°C.

2.5. Statistics

Data pertaining to *S. aureus* were normally distributed and comparisons between test substances and controls were performed using the paired *t*-test. Data pertaining to *P. aeruginosa* were not normally distributed and comparisons between test substances and controls were performed using the Wilcoxon signed rank test. Statistical significance was regarded as $P < 0.05$.

3. Results

3.1. Anti-elastase activity of elafin moieties

All three peptides (full-length elafin, the NH₂-terminal domain and the COOH-terminal domain) were tested for anti-HNE activity (Fig. 1). No anti-HNE activity was detected in the NH₂-terminal domain. In contrast, the COOH-terminal domain and the full-length molecule were found to have an identical anti-HNE activity, indicating that elafin's anti-elastase

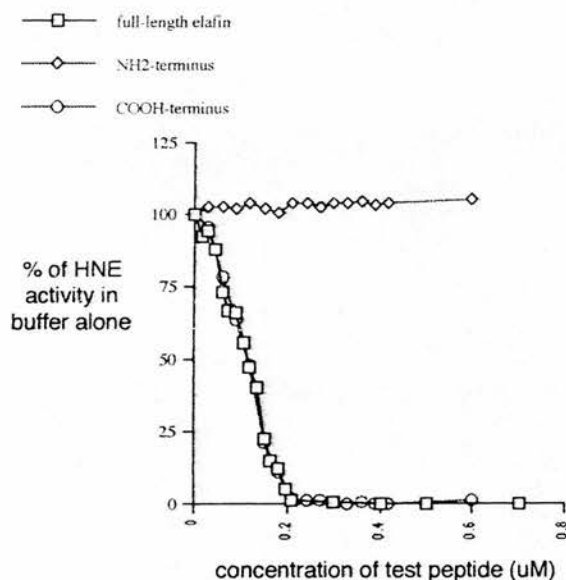


Fig. 1. Anti-elastase activity of full-length elafin, NH₂-terminal domain elafin and COOH-terminal domain elafin. Known quantities of test inhibitor were added to 300 ng HNE. All dilutions were performed in Tris 50 mM, Triton 0.1%, sodium chloride 0.5 M, pH 8.0. Chromogenic substrate (*N*-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide) was added and the change in absorbance at 405 nm was measured as a function of time. The molecular weights of full-length elafin, NH₂-terminal domain elafin, COOH-terminal domain elafin and HNE are 9.9, 5.2, 4.8 and 30 kDa, respectively. Results are expressed as a percentage of the HNE activity when incubated with buffer alone.

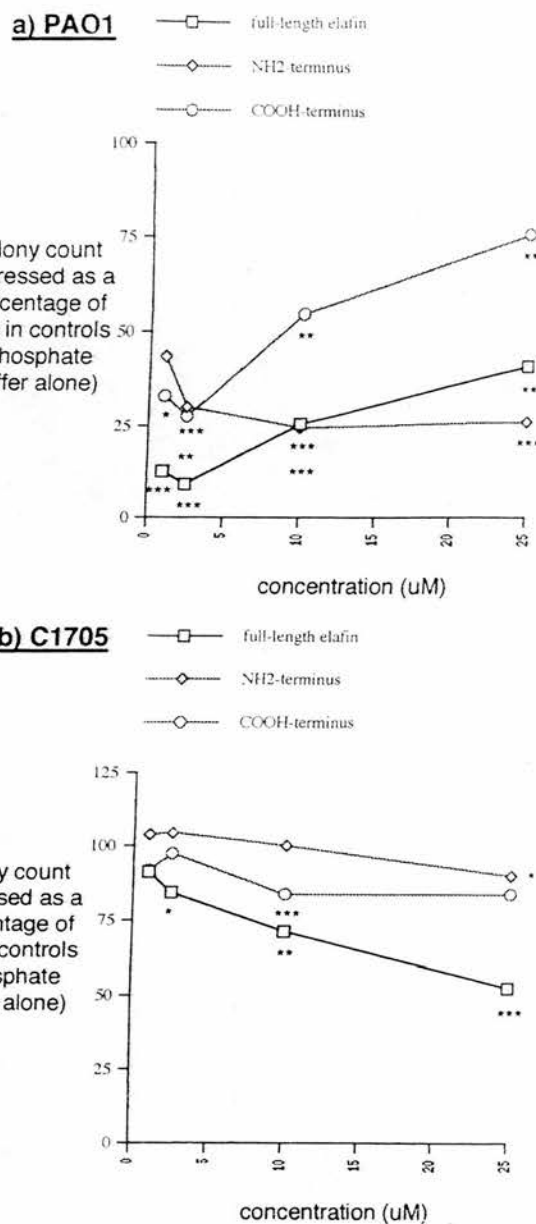


Fig. 2. Anti-microbial effect of elafin moieties against *P. aeruginosa* (PAO1) and *S. aureus* (C1705), expressed as a percentage of the colony count in phosphate buffer alone (control). In (a), results represent medians ($n=8$ for concentrations of 2.5–25 μ M; $n=4$ at 1 μ M). In (b), results represent means ($n=5$). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

tase activity resides exclusively in the COOH-terminal domain.

3.2. Anti-microbial activity of elafin

Full-length elafin resulted in a significant killing of PAO1 at all doses tested (1–25 μ M) (Fig. 2). The maximum effect was observed at 2.5 μ M, at which 93% of PAO1 was killed, relative to PAO1 grown in phosphate buffer alone. At 1 and 2.5 μ M, the contributions of the NH₂-terminus and COOH-terminus were approximately additive, but at higher doses, the predominant anti-microbial effect resided in the NH₂-terminal domain. The anti-microbial effect of full-length elafin against

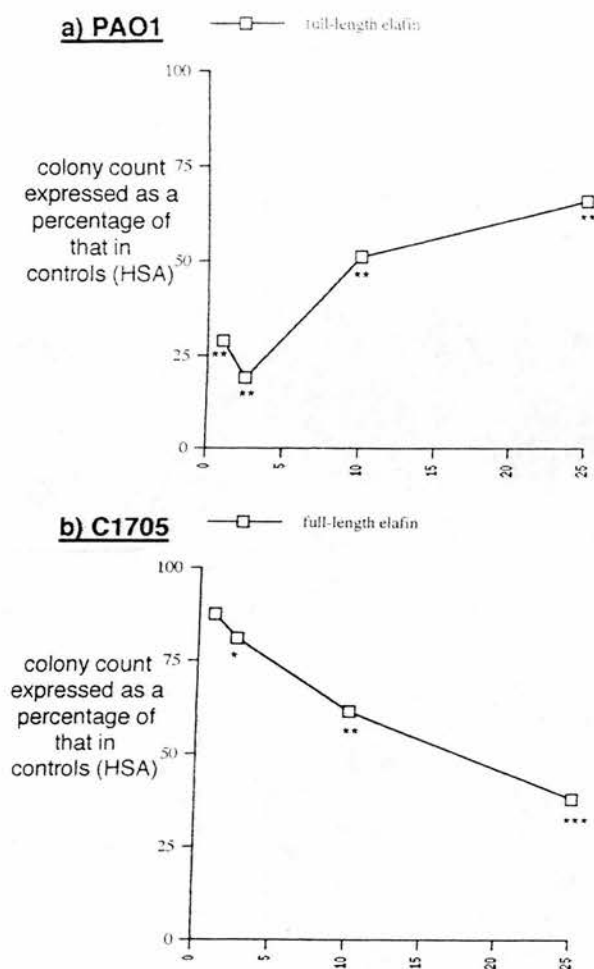


Fig. 3. Anti-microbial effect of full-length elafin against *P. aeruginosa* (PAO1) and *S. aureus* (C1705), expressed as a percentage of the colony count in HSA. In (a), results represent medians ($n=8$ for concentrations of 2.5–25 μM ; $n=4$ at 1 μM). In (b), results represent means ($n=5$). Comparisons were made between equimolar concentrations of test peptide and HSA. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. For reasons of visual clarity, only data for full-length elafin are shown, however, dose-response curves for NH_2 -terminal domain elafin and COOH-terminal domain elafin also closely paralleled those shown in Fig. 2.

S. aureus (C1705) was dose-dependent and was significant over a dose range of 2.5–25 μM (Fig. 2). At 25 μM , 48% killing of C1705 was achieved. The NH_2 -terminus showed a significant anti-microbial activity only at 25 μM and the COOH-terminus only at 10 μM . To ensure that the effects observed were not attributable to a non-specific peptide effect, the effects of full-length elafin, the NH_2 -terminal domain and the COOH-terminal domain against PAO1 and C1705 were also compared with a control protein (HSA) (Fig. 3). For full-length elafin, the maximal activity was described at 2.5 μM against PAO1 (81% killing) and at 25 μM against C1705 (63% killing) (Fig. 3), closely in keeping with the findings when the effect of full-length elafin was compared with that of phosphate buffer alone (Fig. 2). The dose-response curves generated for both NH_2 -terminal domain elafin and COOH-terminal domain elafin against PAO1 and C1705 also closely paralleled those obtained when survival was expressed relative to PO_4 buffer instead of HSA (data not shown).

3.3. Anti-microbial activity of SLPI

In a separate set of experiments, the activity of SLPI against PAO1 and C1705 was tested over the dose range 1–10 μM and compared with that of full-length elafin (Table 1). The anti-microbial activity of elafin against PAO1 conformed to the characteristic pattern shown in Fig. 2, with a maximal effect at 2.5 μM . The anti-microbial activity of elafin was greater than that of SLPI at all doses tested. To test for potential synergy, equimolar concentrations of SLPI and elafin (0.5 μM elafin added to 0.5 μM SLPI) were compared with either 1 μM elafin or 1 μM SLPI alone. No evidence of a synergistic effect was found (86% killing of PAO1 using the elafin/SLPI combination, 88% using elafin alone, 80% using SLPI alone). The anti-microbial activity of elafin and SLPI against C1705 was similar over the dose range tested.

4. Discussion

Elafin (ESI/SKALP) was originally characterised and sequenced from human bronchial secretions and from human psoriatic skin, on the basis of its anti-elastase activity [9,14]. The identification of anti-microbial activity against Gram-negative and Gram-positive respiratory pathogens at concentrations potentially achievable in epithelial lining fluid [28] (especially after genetic augmentation [26]) suggests more complex functions for elafin in the context of inflammation. This observation is in keeping with the identification of additional functions for other major anti-elastases such as SLPI, which has intrinsic anti-bacterial and anti-viral activity [5,16,29] and which can influence the function of lipopolysaccharide (LPS) [30] and prostaglandins [31].

Indeed, elafin can be added to the growing list of endogenous lung peptides which harbour an anti-microbial function, including defensins, SLPI, lactoferrin and lysozyme [1,6]. These molecules share certain general characteristics, for example, a low molecular weight and net positive charge. However, each has unique structural features, which may play a role in determining the anti-microbial function. The gene sequence and derived amino acid sequence of elafin have allowed identification of various structural determinants [13]. The NH_2 -terminal domain, as defined in this study, has a net positive charge of +5, as well as repeated structural motifs potentially acting as substrate for transglutaminase which may allow elafin to bind to the interstitium covalently [13,15,32]. The COOH-terminal domain, as defined in this study, has a net positive charge of +2 and has four disulfide bonds which confer structural stability [13,32]. Previous studies using elafin fragments of either 57 amino acids (H_2N -

Table 1
Anti-microbial effect of full-length elafin and SLPI against *P. aeruginosa* (PAO1) and *S. aureus* (C1705)

	1 μM	2.5 μM	10 μM
PAO1			
Full-length elafin	95.7	96.3	85.1
SLPI	93.8	94.7	73.1
C1705			
Full-length elafin	–9.8	–7.9	13.6
SLPI	–8.1	18.1	6.0

Results are expressed as percentage killing relative to that in PO_4 buffer alone (taken as 0%) and represent medians from three separate experiments.

When expressed relative to HSA, the results obtained were similar and followed the same trend (data not shown).

14 AQE..... 14 Q-OH) or 48 amino acids (H₂N- 14 STK.....Q-OH) have demonstrated high affinity anti-elastase activity (K_i in the nM range), but were unable to provide information concerning the NH₂-terminal end of the molecule [9,14,33]. These findings, in conjunction with crystallographic studies [17], suggested that elafin's anti-elastase activity resides exclusively in the COOH-terminal domain. Using discrete functional domains of elafin and studying full-length elafin for the first time, we have been able to provide direct confirmatory evidence to support this hypothesis by demonstrating that the COOH-terminal domain of elafin and the full-length molecule bind HNE with an equal efficiency (Fig. 1).

Furthermore, the use of discrete functional domains of elafin has allowed us to infer which regions of the molecule may be responsible for anti-microbial effects. In the case of *P. aeruginosa*, our data suggest that inherent anti-microbial activity exists in both structural domains and imply that their interaction may be necessary to augment anti-microbial activity at low concentrations. The inverse dose-response relationship noted may reflect the ability of *P. aeruginosa* to use higher concentrations of peptide as nutrients, promoting growth and competing with the killing effect of elafin. Indeed, *P. aeruginosa* is known to grow in increasing concentrations of organic nitrogen [34]. This may explain why elafin's anti-microbial effect was slightly less pronounced when albumin was used as control (see Fig. 3).

The anti-microbial effects of several low molecular weight peptides have been ascribed to their cationic nature. Cationic peptides are capable of binding *P. aeruginosa* LPS, altering the outer membrane stability and increasing susceptibility to other bactericidal compounds [35]. While the charge may have contributed to the killing observed here, it is unlikely to explain the entire anti-microbial effect of elafin (especially at low doses, where the less cationic COOH-terminal domain was more effective).

The anti-microbial activity against *P. aeruginosa* was independent of the anti-elastase activity of elafin, as the majority of anti-microbial activity resided in the NH₂-terminal domain, while anti-elastase activity resided exclusively in the COOH-terminal domain. Interestingly, a quite different, dose-dependent pattern of anti-microbial activity was seen against *S. aureus*. The NH₂-terminal domain alone contributed only slightly and at high concentrations. As with *P. aeruginosa*, this suggests that a simple charge effect is unlikely to explain elafin's anti-microbial effect. The observation that full-length elafin was more effective than the additive effects of the two structural domains may again imply a critical interaction between these. In a similar study using structural domains derived from SLPI, Hiemstra et al. [5] also found that the full-length molecule was more active against *Escherichia coli* and *S. aureus* than was either terminal fragment.

In our hands, SLPI was found to have anti-microbial activity against *P. aeruginosa* and *S. aureus* as has been described elsewhere [5,16]. The anti-microbial effects of SLPI were less pronounced in our study, but this may reflect the use of different clinical strains. Our data suggest that in equimolar concentrations, elafin is at least as effective as SLPI against PAOI.

The anti-microbial activity of elafin adds to the emerging picture of its primary role in the lung defence. Elafin is ideally placed to promote the early eradication of invading pathogens and to protect the host against proteolytic destruction in the

event of neutrophil recruitment. Indeed, the co-existence of anti-microbial and anti-elastase activity in the elafin molecule could have therapeutic implications. *P. aeruginosa* and *S. aureus* can each cause severe pneumonia [18,21] and frequently co-colonise patients with cystic fibrosis (CF) [19]. The significant morbidity and mortality associated with these organisms, coupled with their propensity to develop resistance to conventional antibiotics [22,23], demands the development of novel anti-microbial strategies. In CF, HNE is thought to contribute to the airway pathology by degrading substrate in the interstitium, enhancing inflammatory cell chemotaxis, stimulating mucous hypersecretion and promoting the adherence of *P. aeruginosa* [36–38]. Furthermore, PAOI is known to promote significant release of elastase from hamster neutrophils in vivo [39]. Effective augmentation of anti-microbial anti-elastases for patients known to be at risk of developing infection with *P. aeruginosa* and/or *S. aureus* may thus be theoretically desirable. Our findings suggest that elafin gene augmentation could be particularly beneficial in CF. Elafin levels are known to be reduced in CF [40], high levels of elafin can be effected using adenoviral gene therapy in rats in vivo [26], elafin's transglutamination sites may confer a longer biological half-life in vivo [15,32] and elafin appears particularly active against the non-mucoid clinical isolate PAOI. Strategically, eradication of *P. aeruginosa* whilst still in the non-mucoid form may be especially important in preventing or delaying progression to chronic infection with mucoid variants, which are seldom eradicated and are associated with a significantly worse prognosis in CF [20].

We recently showed that genetic augmentation of elafin using adenoviral gene therapy protects human alveolar epithelial (A549) cells against HNE and activated neutrophils [41]. We have extended these findings to show that elafin's anti-microbial activity against PAOI can be augmented in supernatant derived from A549 cells transfected with adenovirus encoding elafin (unpublished data). This suggests that genetic augmentation of endogenous anti-microbials may be effective against pulmonary pathogens, as has also been demonstrated using γ -interferon [42,43].

In summary, these findings demonstrate for the first time that elafin has an intrinsic anti-microbial activity against important Gram-negative and Gram-positive respiratory pathogens and that this activity is independent of the molecule's anti-elastase activity.

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